

## Chip war about to break out again?

*The 1986 agreement between the United States and Japan on the supply of microchips seems to violate the rules of free trade. They should look to a more lasting solution of the problem.*

THE European Commission is understandably delighted with the impending ruling of the council of the General Agreement on Tariffs and Trade (GATT) that the agreement between the United States and Japan on the supply of microchips violates the rules of international trade. That, no doubt, is why the commission chose last week (24 March) to make public the news that GATT's disputes panel has upheld its own complaint about the agreement, reached after tense and tortuous negotiations in the summer of 1986. Apparently the GATT panel agrees that, by imposing lower limits on the prices at which Japanese chips are sold in third markets (Europe in particular), the agreement constitutes a cartel inimical to the principles of free trade. For what it is worth, the panel does not accept the commission's argument that the chip agreement discriminates against European manufacturers by its provisions for giving US suppliers access to Japanese markets. The council of GATT will have to decide whether or not to ratify the panel's finding at its next meeting on 4 May, but whatever happens is certain to be embarrassing.

Even the European Commission will in some ways seem foolish. While celebrating its victory in the GATT committee, the commission is also busily investigating complaints by European chip producers that Japanese companies have been selling some kinds of chips at uneconomically low prices in Europe ("dumping"), suggesting that there cannot be much substance in the complaint that the US-Japanese agreement has actually deprived European users of microchips of the benefits of cheap Japanese products. In reality, the situation is more complicated. There are chips and chips, and it is entirely possible that some may be overpriced and others underpriced. And there is ample substance in the commission's argument that the prices at which Japanese manufacturers sell chips to European customers should be fixed in Washington under an agreement whose objective is to protect US manufacturers from the full force of Japanese competition. That is the point GATT seems ready to accept.

The embarrassment for the government of Japan will take a different form. The agreement with the United States was reached reluctantly, after a long period of recrimination and at pistol-point (the threat that the US president might not veto restrictive trade legislation the Congress seems always on the point of passing). Now, if the GATT ruling is ratified on 4 May, Japan will be asked to modify the agreement, but not to abandon it (which it would, no doubt, prefer). The obvious difficulty is that there will then have to be another round of delicate negotiations with the chip producers of Japan, most of whom consider the agreement with the United States to be unjust.

But the most serious embarrassment will be that of the US government, whose original advocacy of the chip agreement squares awkwardly with its justifiable reputation as the West's most consistent advocate of free trade. Two years ago, the chip agreement was held to be an indispensable means of keeping at bay an unstoppable tide of imported microelectronics from Japan. The agreement has functioned almost too well; for some months, US users of simple silicon memory chips have been crying out for more, and losing valuable sales because they cannot get enough. Yet since the agreement was signed in 1986,

trade relations between Japan and the United States have been enlivened by troubles, such as Toshiba's breaking of the US strategic embargo by allowing Soviet manufacturers of submarine propellers access to superior milling technology. It will be galling for the United States now to have to plead with Japan to modify an agreement originally accepted only reluctantly. Mercifully, even the Toshiba affair has not permanently soured relations between the two, while the improving US external trade balance may make it easier to reach a new agreement.

The enduring misfortune in this prolonged argument about the price of microchips is that no remedy for immediate problems can be lasting. The United States has two legitimate interests in the affair, of which the simpler is that its manufacturers should not become so dependent on Japanese sources for the supply of chips that its long-term strategic interests will be undermined. As things are, the US Department of Defense contracts for the supply of microelectronic equipment from predominantly US suppliers (which is no offence to GATT), supports research and development in the field on a huge scale and takes care that US manufacturers (such as Fairchild last year) about to be taken over by Japanese companies are kept, by administrative action, in US hands. In the medium term, over a decade or so, these devices are probably enough to ensure the strategic interest of the United States. They are expensive devices, but they should suffice. If, on a much longer timescale, Japanese or other manufacturers should gain an even more striking advantage than they have at present, that could be worrying, but so then would the general economic position of the United States from which its strategic strength flows.

### Unfair?

The second legitimate US interest is that trade with Japan should be fair in some appropriate sense. There has been a temptation in the United States to argue that the trade in microelectronic products is manifestly unfair because, at least until the fall of the dollar against the yen beginning last November, Japanese products were cheaper than could be economically produced domestically. But the logic of that argument is strained at best.

What, for example, if Japanese manufacturers are inherently more efficient than their US counterparts in the production of microelectronic components and the machines assembled from them? That this may be the case is borne out by the success of Japanese manufacturers in other markets over many decades, and by what is known anecdotally of Japanese production technology. But in the long run, and if microelectronic products were a substantial and decisive part of mutual trade, the only remedy might be that recently forced on the United States — the depreciation of the currency. Luckily, there is another side to the coin; Japan, lacking an aircraft industry, buys almost all its civil and military aircraft from the United States.

US complaints that Japan protects domestic industries by an array of non-tariff barriers, widely echoed in Europe, have some substance, but less than might appear. In the recent rows about the supply of telecommunications equipment for the Japanese internal and external networks, about agricultural produce



and contracts for building the new Osaka airport, the West has been asking Japan for trade concessions not required under international agreements such as GATT and which many of its own members would not willingly surrender. The simplest and most secure remedy is therefore to negotiate an extension of the GATT agreements to cover these disputed areas of trade. That is the best way of extending to international trading relationships Adam Smith's prescription of an economic division of labour as the most effective way of making a national economy efficient. Luckily, negotiations have already begun for an extension of the GATT agreements in these directions. The United States, which has already been asking for the total elimination of agricultural subsidies over a period of ten years, would be better employed putting energy into these negotiations (called the Uruguay round) than in amending its cosy cartel with Japan on chips. □

## Crypto-proliferation

*The powers might agree on strategic weapons, but what of nuclear proliferation?*

INTEREST in the summit meeting now planned for June in Moscow understandably revolves about the hope that the United States and the Soviet Union will by then have hammered out the basis for an agreement on strategic arms. The familiar hope is that the two superpowers will be able to settle for a 50 per cent reduction of their own nuclear missiles without looking too often and apprehensively over their shoulders at what other people are about. But this may be a lot to ask. Not only does the long-standing problem of British and French nuclear weapons remain unclarified, but worrying signs of mounting nuclear ambitions are apparent elsewhere.

China's naval dust-up with Vietnam the other day should be a reminder that China is also a nuclear power, probably quite a substantial one by now, a quarter of a century after its first nuclear explosion. The conviction last week by Israeli courts of an ex-employee at the Dimona nuclear plant on charges including one tantamount to treason is similarly a reminder that Israel has been a crypto-nuclear power for almost as long. (The accused's sentence has not yet been handed down, but it is unthinkable that he would have been convicted of such serious crimes if his tale, to a British newspaper, of plutonium manufacture at Dimona were entirely a pack of lies.) The advantage of that state of being is that it inspires fear among adversaries without risking the contumely of the major powers. In some eyes, those in Pakistan in particular, India has achieved that status. Its first nuclear explosion was in 1974, while Mr Rajiv Gandhi's statements on India's nuclear ambitions are even more delphic than his mother's used to be; last month's test-firing of a long-range rocket (see *Nature* 332, 9; 1988) will darken sinister suspicions. Whether Pakistan is travelling along the same road is still conjectural, but could be dismissed only rashly.

Those planning to travel to Moscow in June should take care that these side-shows to their main business are not entirely overlooked. Nuclear proliferation has not been and is not likely to be as rapid as the pessimists would insist, but there is more than enough crypto-proliferation to be worrying. It is especially alarming that the three powers (Israel, India and Pakistan) to which the label is attached either have interests in the powder-keg of the Middle East (Israel and Pakistan) or are likely to be affected by forthcoming events, such as the intended withdrawal of Soviet troops from Afghanistan. The summit powers have a long-standing interest in preventing proliferation, and should be prepared to use their influence to bring China and France within the Non-Proliferation Treaty as nuclear powers. The objective should be to bring all this about no later than the next review conference of the treaty, due in 1990. Then there might be a reasonable chance of dissuading the cryptonuclear powers from going further down this dangerous road. □

## Fighting wrong battles

*The US science community risks blaming the messenger for bad news about the NSF budget.*

THE US National Science Foundation (NSF) is well on the way to being the victim of its own success. Although regarded in Washington as the voice of the US community of scientists, especially those in small universities whose careers revolve around small experiments and modest grants, NSF may elsewhere be seen as an enemy — the agent of an unsympathetic administration whose purpose is mainly to turn down grant applications and deny funds to senior scientists on whimsical grounds after years of favourable peer review. So much was clear at this year's Spring meeting of the American Physical Society at New Orleans, where suspicion of NSF abounded. Alvin Trivelpiece, formerly of the Department of Energy and now with the American Association for the Advancement of Science, was right to say that the reaction of the US physics community to the shortage of funds has been to "form the wagons into a circle and start shooting inwards". That the frustration of scientists is directed at NSF, not at the White House or the Congress, is one of the reasons why the frustration continues.

People are understandably bewildered that NSF has emerged from last year's budget deliberations with essentially level funding. Did not the move to increase NSF spending by 30 per cent this year, with the prospect of doubling in five years, sail through Congress with only three dissenting votes? Many politicians may oppose particular research projects, but none opposes science in general. All agree that science is important for technological progress and industrial competitiveness, this year's political battlecries. But what the science community has failed (or refused) to understand is that, when hard decisions are made, benevolent feelings cannot compete with immediate interests. No congressman fears for his seat because physicists might speak out against him.

Many have taken the debacle over the NSF budget as yet more evidence of the essential shiftiness of politicians, and have walked away from the whole shabby business, grumbling disdainfully. That is why so many have turned against NSF, complaining about unjustifiable spending on Big Science rather than Small Science, or pure rather than applied research, or cosmology rather than solid-state physics. All this is cheering for the farmers', the veterans' and the military lobbies who are confirmed in their belief that the science community is divided and thus unlikely to threaten their portions of the federal pie.

Solid-state physicists, semiconductors, superconductors and the like notwithstanding, consider themselves especially unappreciated. While their counterparts in big-spending areas such as astronomy and particle physics have long recognized that projects costing hundreds of millions of dollars will not be supported simply out of intellectual curiosity, the solid-state community has assumed that the self-evident utility of its work would assure its own support. They are now aggrieved that they must also demonstrate the practical benefits of their science as compared, for example, to building giant telescopes or particle accelerators.

What solid-state physicists and others in 'small science' must do is not to denounce their big science colleagues as bullies or charlatans, but to promote the evident virtues of their own fields. This should require little more than careful explanation of what they are about — and of why they are worried that the Japanese are moving faster. But it does require that scientists make an effort to understand how and when federal dollars are shared out. They must learn, distasteful though it may be, what other forces they are competing against. They must also guard against a failing seemingly common among physicists — the suspicion that becoming involved entails telling other people what to do. □



# ESA's science programme will fail without Britain

- Britain: not a penny more
- Twenty-year timescale critical

## Paris

IF Britain continues to veto a 5 per cent annual increase in the mandatory basic science budget of the European Space Agency (ESA) beyond 1989, the entire Horizon 2000 science programme could be doomed, says Professor Roger Bonnet, ESA's director of science. Since the addition of three new member states last year — Finland, Norway and Austria — it is not so much Britain's financial contribution that is crucial, says Bonnet, but the fact that the UK veto overrides the otherwise unanimous support for the increase from the remaining 13 members.

At ESA's ministerial meeting in the Hague last November, Mr Kenneth Clarke, Britain's Minister of State for Trade and Industry, said that Britain would give "not a penny more" to ESA (see *Nature* 330, 195; 1987). Having seen Britain confirm its stance at the ESA Council meeting in December 1987 (see *Nature* 330, 594; 1987), the Science Programme Committee met on 22 March to look for ways to save Horizon 2000.

Rather than maintain the current 5 per cent annual increase in the science programme budget, Clarke suggested that the programme's activities could be spread over a longer period, but a Science Programme Committee memorandum unanimously endorsed at last week's meeting — Britain abstained — argues that the 20-year timeframe corresponding to "one generation of scientists", is critical.

Horizon 2000's "minimum balanced plan" of mission sizes and disciplines, established in 1984, received unanimous approval from member states, including

Britain, at the 1985 ministerial meeting in Rome. "Horizon 2000", says Bonnet, "has become a reference plan for space scientists both within and outside Europe."

But the original 1985 timetable was upset by the loss of both the US Challenger shuttle and of Europe's Ariane



ESA's Roger Bonnet, who played a key role in the 1986 Giotto mission to study Halley's comet, named 1987 Science Personality of the Year by an international committee of previous recipients.

rocket launcher in 1986. The Ulysses solar power mission and the Hubble Space Telescope, which rely on the shuttle, have both to be postponed until late 1989 or 1990. And the Hipparcos space astrometry mission, originally due to be launched by Ariane 4 in July this year, will now have to be kept in storage until spring 1989.

The delays will cost ESA an estimated 918 MAU (million accounting units; 1 AU

## Pan American satellite gains access to UK

### Washington

THE UK Office of Telecommunications (OfTel) has cleared the way for the US company Pan American Satellite to offer services to British customers. PanAmSat had petitioned OfTel for relief, claiming that British Telecom was unfairly refusing to provide downlinks for PanAmSat to tap into the UK market.

Earlier this year, PanAmSat launched a media campaign to highlight its problems in gaining landing rights in the United Kingdom (see *Nature* 331, 294; 1988). International telephone switching services are the exclusive province of Intelsat, but companies such as PanAmSat wish to offer

other services, such as data transmission and dedicated video and audio lines that lie outside Intelsat's jurisdiction, and must get local telephone companies to provide downlink and ground-based services.

Professor Bryan Carsberg, OfTel's director general, in response to a PanAmSat complaint, ruled that British Telecom was obliged to provide downlink services to PanAmSat so long as there was no breach of its licence conditions. PanAmSat praised the ruling, calling it the first breakthrough to a European market.

PanAmSat's satellite PAS I is scheduled to be launched by an Ariane rocket on 23 May this year. Joseph Palca

## Cluster and Soho go-ahead

### Paris

IF the European Space Agency (ESA) Horizon 2000 science programme is down, (see below) it is not yet out. At last week's meeting of the agency's Science Policy Committee the go-ahead was given for a new Solar Terrestrial Science Programme (STSP) to be financed two-thirds by ESA and one third by the National Aeronautics and Space Administration (NASA).

STSP is made up of two space missions, involving "an armada of satellites" and around 500 scientists in Europe and the USA. "Soho" — the Solar and Heliospheric Observatory — will orbit the Sun at a constant distance of 1.5 million kilometres from the Earth in order to record observations of the solar surface, corona and solar wind, and will be able to study the effects of solar activity on Earth's magnetosphere.

The second programme, "Cluster", will involve four identical spacecraft orbiting in formation to investigate interactions between the solar wind and the plasma environment of the Earth.

Both missions will be launched in the mid-1990's, one each by ESA and NASA, and are expected to operate for over two years.

Peter Coles

= £0.63 at 1986 exchange rates) by the end of the 20-year duration of the programme.

If the shortfall is not made up, says Bonnet, "two of the main missions will have to be excluded from the 20-year programme. If the X-ray mission is delayed by 5 or 6 years it will lose its competitiveness and will set back the whole domain of X-ray astrophysics... The statement by Mr Clarke that Horizon 2000 can be extended without damage is simply not true — it will be destroyed."

The Science Programme Committee is determined not to let this happen and last week resolved to "urge the council to take the necessary political steps to ensure that the Long Term Plan Horizon 2000 is preserved". But what are the options?

It may be possible to use the recent addition of contributions from Finland, Norway and Austria (which total about 5 per cent of the budget) to offset current losses caused by the delays, but, so far, this has been agreed only for 1987 and 1988. In 1989, the extra contributions would normally be offset by a corresponding decrease from other members.

The 5 per cent annual increase from Britain amounts to only £1.1 million. "If Britain were to leave ESA and the other member states increased, we would be better off", says Bonnet, "but she is a key member. This is not what we are asking".

Peter Coles



## UK nuclear power research squeezed by market forces

### London

THE United Kingdom Atomic Energy Authority, whose main business is research and development in support of the nation's nuclear power programme, has emerged from its second year of commercial operation with the announcement that it is to shed between 70 and 100 jobs at one of its eight laboratories. The authority, which employs around 14,000 people and has an annual turnover of some £400 million, became a trading fund in early 1986, having previously been government-funded through a grant-in-aid. The authority's income is now derived exclusively from contracts, although the Department of Energy and the Central Electricity Generating Board (CEGB) remain the principal customers.

The authority's first year as a trading fund was seen through successfully, with the financial targets set by the government having been achieved. To do this the authority deliberately broadened its commercial base by expanding its non-nuclear research capability, thereby increasing its turnover by 15 per cent to £38.4 million — 9.2 per cent of total turnover.

Market forces are, however, now taking their toll. In October last year, Stuart Nelson, director of the authority's Northern Research Laboratories, launched a review of the laboratories' commercial future as part of a wide-ranging reassessment of the authority as a whole. The principal recommendation of Nelson's review, which was formally adopted on 17 March, is to run down activities at the Springfields laboratory in Lancashire, which is largely involved with the 'back end' of the fuel cycle, developing routes for dismantling fast reactor and other fuel elements. It is also the authority's centre for work on power fluidics, developing devices without moving parts for handling large volumes of active fluids in future plants such as the Thermal Oxide Reprocessing Plant at Sellafield, and houses the Structural Integrity Centre, which carries out fatigue and fracture testing of reactor components and other materials.

Much of Springfields' work will now be concentrated at the Northern Laboratories' other two sites at Risley and Windscale. The changes are necessary, says the authority, to reduce costs and to "match an expected decline in income and to become more competitive in seeking new business". The run-down will be phased over three years. The authority says the decline in income will arise mainly because work will become redundant as Magnox reactors approach the end of

their lives, advanced gas-cooled reactors cease to be built and prior specification work on pressurized-water reactors has been completed.

However, another aspect of the authority's future income remains less predictable. As the CEGB prepares to be split into two parts and sold to private investors, the future of its nuclear research programme is far from clear. Already this year the CEGB has withdrawn from two Atomic Energy Authority projects. In January, the board announced that it would no longer contribute to the building of an £18 million laboratory for post-irradiation examination. Soon afterwards, at the end of February, the board said that from April it would withdraw from a 15-year, £50 million programme to dismantle the 33-MW advanced gas-cooled reactor at Windscale. The board had agreed to contribute £25 million to the scheme and had already committed £5 million. The Atomic Energy Authority is now seeking new partners.

Simon Hadlington

## Superphénix reactor set to rise again?

### Paris

THE French prototype fast-breeder reactor, Superphénix, which was shut down last year when a storage cylinder containing liquid sodium coolant developed a leak (see *Nature* 328, 100; 1987), may be able to start again in October this year.

The plan, put to industry minister Alain Madelin, by engineers from Electricité de France (EDF), involves replacing the liquid sodium with inert argon gas. As the hair-line cracks causing the leak have been found only in the inner liner of the cylinder (see *Nature* 331, 471; 1988) the outer shell would be sufficient to maintain a sealed environment for fuel rods in transit to and from the reactor core, the experts say.

The cost of switching to an argon coolant is estimated at FF300 million (£30 million), compared to over FF400 million if the cylinder were to be replaced. But a change may have to be made in the procedure for operating the reactor. Fuel rods would have to be replaced only every three or four years, rather than annually as before, an operation which could itself take up to a year.

A final decision will not be taken until early summer, after safety experts have studied thousands of X-ray photographs of the cylinder and have carried out further tests.

Peter Coles

## German centre for California

### Berkeley

THE West German Ministry for Research and Technology has decided that California is the place for computer science. In a matching-funds arrangement with a consortium of German industries, it has provided for the establishment of the International Center for Computer Science (ICSI), an independent research institute located near the University of California (UC) campus at Berkeley.

Although German computer science is already well-supported at the German National Center for Computer Science and several other university-associated laboratories, the money spent on ICSI is a concession to the reality of US leadership in computer science, and recognition that a US institute is more likely to draw the best international talent than one located in West Germany. The Germans will be rewarded for their effort with an international training ground for their young scientists, and the opportunity for collaboration on all levels, which they expect ultimately to enhance the quality of their science at home. The institute will focus on parallel computation, and will be headed by Jerome Feldman, recently appointed to the UC Berkeley faculty from the University of Rochester.

Originally established in 1986 with less than \$1 million in German seed money, ICSI operated with just a handful of staff until this month, when it moved to 14,000 feet of renovated office space. The Germans have pledged support approaching \$5 million a year for the next 5 years, renewable upon review. That will support some 30 scientists, as well as graduate students and postdoctoral researchers, says Feldman. The institute hopes to encourage other governments to be members and to bring in additional funding through US government research grants.

The sponsors do not have control over ICSI's direction, but German scientists hold two positions on the 9-member board of trustees. In addition, West Germany and any other supporting countries are likely to be more heavily represented among post-doctoral fellows, said Feldman. The institute has set up a competitive fellowship programme in West Germany, and expects to take up to 8 German postdoctoral fellows in the first year.

Research at ICSI will be 'pre-competitive' basic research, said Feldman, adhering to the institute's charter. His goal is to bring together computer scientists from diverse disciplines, as well as connectionists with backgrounds in biology, linguistics and psychology, to address the problems of parallel computing.

Marcia Barinaga

# Trade union reform in the offing for Hungary's scientists

London

THIS week, some 2,000 Hungarian research institutes, laboratories and scientific establishments have received letters setting out the principles of the proposed new Democratic Trade Union of Scientific Workers. The letter asks scientists and researchers to comment. But officials of the National Trade Union Council (SZOT) have already made their views clear: by drawing Hungary's 77,000 scientists and scholars into a single union, they say, the new body could put at risk the unity of the trade union movement in Hungary.

Hungarian trade unions are organized on the basis of workplace, not profession, so that Hungarian scientists are spread over 16 unions, while in a given institute everyone from scientific director to janitor comes under the same union. During the past few weeks, there has been discussion among the members of the founding committee of the new union as to whether to stay within the SZOT confederation, or whether the new union should become the nucleus of a new profession-related trade union structure. It is this more radical proposal that alarms SZOT.

In a recent radio interview, Csaba Oeri, one of the founding committee of the new union, said that the situation of scientists and scientific research in Hungary has been "devalued" over the past few years, and that the possibilities of doing effective research are diminishing. These difficulties, Oeri said, predated the present economic difficulties. Nevertheless, the recent cuts in science spending in Hungary may have played some role in triggering the initiative. As the president of the Academy of Sciences, Dr Ivan T. Berend, warned last autumn, the cutback could lead to the closure of institutes and research groups. And, as Oeri pointed out, the new

union would not only look after scientists in employment but also those who lose their jobs.

The scientists' main criticism of the existing union structure is that clashes of different interest groups within a given union make it extremely difficult for the interests of any to be properly represented. The founding committee, said Oeri, supported the idea of "a much more direct and original interest representation" with greater independence for the individual basic organization.

The new union could not, Oeri admitted, form part of SZOT under the latter's present statutes. But the founders, he said, are taking seriously the frequent assertions by SZOT officials that the trade union movement is living in an age of renewal.

The scientists' demands for greater democracy and grass-roots power in the union movement has received some sympathy from Endre Szabo, general secretary of the Trade Union of Public Employees, which includes among its members some 8,000 scientists (including those working in institutes). Although "not convinced" of the need to establish a separate trade union for scientists, he recommended that his union should adopt a "federative" structure, in which the scientists would form an autonomous unit, with complete independence in dealing with matters concerning research institutes. The reactions of the would-be founders of the Democratic Trade Union of Scientific Workers to these compromise proposals have been mixed.

**Vera Rich** ■ Warsaw university employees are keeping up their campaign for the re-establishment within the university of the banned Solidarity trade union. The courts have so far rejected 27 petitions for the registration of the union, but the 405-person "founding committee" immediately files another application. While the petition for legalization is before the courts, they say, they have some protection against charges of belonging to an illegal organization.

Dr Janusz Onyszkiewicz, a lecturer in mathematical logic, who during Solidarity's legal existence was the union's principal press spokesman, says that in addition to its 405 members, the committee has "several thousand" supporters within the university, more than belong to the "official" trade union. And the movement is gaining quasi-recognition. Last Friday, the committee members and several hundred supporters were able to hold a meeting in one of the largest halls of the university — with the permission of the authorities.

## CFCs phased out

Washington

THE Du Pont Company, the world's largest producer of chlorofluorocarbons (CFCs) last week announced its intention to phase out production of fully halogenated CFCs. The decision comes in response to the report of the Ozone Trends Panel, a committee of the National Aeronautics and Space Administration, to re-evaluate data on global decreases in atmospheric ozone (see *Nature* 332, 293; 1988). The panel concluded that trace amounts of CFCs in the atmosphere played a role in the stratospheric ozone depletion not explained by sunspot activity.

Du Pont pledged to "continue our aggressive efforts to develop environmentally safe alternatives". J.P.

## US-Japan flight

Washington

AN attempt to force renegotiation of the US-Japan nuclear cooperation agreement failed by a vote of 53 to 30 in the US Senate last week.

The agreement, signed in November last year, has been controversial because it gives Japan blanket approval to reprocess spent US-supplied reactor fuel. As Japan does not have reprocessing plants of its own, the agreement allows for spent fuel to be transported to plants in Europe and for purified plutonium to be flown back to Japan via Alaska. The Senate Foreign Relations Committee earlier voted to recommend that the agreement be renegotiated (see *Nature* 331, 7; 1988).

Opponents of the agreement argue that it loosens US control over the movement of plutonium and thus contravenes the Nuclear Non-Proliferation Act.

The US State Department has agreed that plutonium-carrying planes will not be permitted to fly over Alaska, but will be forced to fly a zigzag route from Europe, up to the North Pole and then down the international dateline to Tokyo, so that the whole flight is above ocean. A.A.

## Merger idea floated

London

THE heads of two of Britain's five research councils are pondering aloud the possible merits of a merger. The financially strapped research councils for Agriculture (AFRC) and the Natural Environment (NERC) point to the overlap of interest in many areas. Advances in many fundamental areas of science, such as molecular biology, have increasingly blurred the traditional divisions of responsibility between the research councils.

Given that the AFRC is to join NERC on the same site in Swindon at the end of the year and that the Advisory Board for the Research Councils is presently examining the research council structure, a change seems likely. S.H.

## Thin-film method



A chemical vapour deposition technique developed by Japanese researchers at Tohoku University and Riken Corporation was used to coat this chrome-nickel coil with yttrium-barium-copper oxide (see *Nature* 332, 295; 1988). The coil winding is 4.5 mm across, the chrome-nickel wire 0.35 mm in diameter, and the superconductor film is about 1 micrometre thick. □



## No to academic freedom clause

London

BRITISH academics are not letting up in their efforts to persuade the government further to amend its proposals on the management of higher education. The government has already bowed to pressure by incorporating several amendments to the Education Reform Bill, soon to go before the House of Lords (see *Nature* 331, 645; 1988). Academics remain adamant that the bill must contain a provision to protect academic freedom upon the abolition of tenure for university staff. The Secretary of State for Education and Science, Mr Kenneth Baker, has consistently maintained that a clause protecting academic freedom would be impossible to word sufficiently precisely, and that the presence of such a provision would render it virtually impossible for an institution to dismiss an academic covered by such protection — that it would be tantamount to tenure.

Last week Baker offered instead to introduce a grievance procedure for dismissed academics, a move that seems likely to cut little ice with the academic community.

The Committee of Vice-Chancellors and Principals (CVCP) is puzzled by Baker's stance (in 1986 the government had no qualms about introducing a statutory right to freedom of speech on university campuses) and is now preparing for its campaign in the House of Lords. In a letter sent to Members of Parliament last week, the CVCP said: "Universities have a number of duties and responsibilities. Among them is to question received wisdom be it religious, political or scientific. Without it our society will be poorer and the international reputation of our universities will be damaged." The CVCP believes it has formulated a definition of academic freedom that would be workable: the freedom within the law to question and to test received wisdom and to put forward new and controversial or unpopular opinions without placing individuals in jeopardy of losing their jobs.

Baker's handling of the lobbyists is causing growing resentment within the Association of University Teachers, which represents more than 31,000 rank-and-file academics. Baker has been talking with the CVCP (albeit to no apparent avail) and has given assurances that he will continue discussions with vice-chancellors to evolve a form of grievance procedure, but has so far ignored the AUT. The AUT has condemned the discussions as "utterly useless" unless the voice of the "ordinary staff" is heard, and rejects the offer of a grievance procedure as "wholly inadequate".

Simon Hadlington

## South Africa uses deportation in the battle against AIDS

Oxford

MORE than 1,000 migrant workers from Malawi, Uganda, Zambia and Zimbabwe are being deported from South Africa because they have been found to be carriers of HIV (human immunodeficiency virus), the Minister of Health and Population Development, Dr Willie van Niekerk, disclosed in Cape Town last month. This follows the introduction of compulsory AIDS screening for all black immigrant workers before they are given contracts. While claiming that its policy is in line with the United States and China, which require foreign immigrants to be screened for HIV, South Africa does not require AIDS screening for white immigrants (7,953 immigrants entered the country last year, most of them white). Nor does it require HIV testing for foreign students, as do the Soviet Union and India.

The incidence of AIDS in South Africa is low (only 2.24 cases per million), and has so far been predominantly confined to white homosexual males. Of the 76 cases reported to the country's AIDS advisory group by the end of last year, 65 were either homosexual or bisexual, three were haemophiliacs, three were related to blood transfusion, and five were contracted by heterosexual contact. Only three of all these cases were black, and only one female; a black woman who contracted

the disease from a blood transfusion.

The advisory group predicts that the prevalence of AIDS is likely to increase more rapidly among black South Africans, and that the pattern of the disease in the future will come to resemble that of the rest of Africa, rather than retaining its present Western character. At least 2,500 cases are expected by 1991. Already, 20 pregnant women, all black, among 37,920 black and white women who underwent routine antenatal tests between May and November last year, have been found to carry HIV-antibodies.

Potential for AIDS transmission is feared to be particularly high among migrant workers in South Africa's mines, where most of workers live in single-sex hostels. There are about 300,000 foreign migrants working legally in South Africa (mostly from Malawi and Botswana), although total estimates (including illegal workers) are closer to a million. About 6.6 per cent of those screened from Malawi have been found to be HIV-positive. The plea of the medical director of the country's blood transfusion service, Dr Maurice Shapiro, that "we should be tracking down those with the infection, counselling them and offering protection against discrimination and prejudice in the workplace", appears to have fallen on deaf ears, at least with regard to foreign migrant workers.

Michael Cherry

## HIV testing for NIH laboratory workers gets under way

Washington

THE US National Institutes of Health (NIH) will offer serologic testing every four months to employees whose jobs routinely expose them to human immunodeficiency virus (HIV), the virus causing AIDS. NIH have identified approximately 200 workers in some 75 different laboratories who qualify. Robert W. McKinney, director of the NIH division of safety, says the programme, which starts next week, is voluntary, and any employees declining to participate will still be eligible for all health benefits.

Employees will be bled at four-month intervals, and their blood will be tested for antibodies to HIV using a commercially approved ELISA (enzyme-linked immunosorbent assay) test kit. Any positive or indeterminate ELISA result will be followed up with a Western blot, or in some cases experimental testing procedures not yet approved by the Food and Drug Administration, such as a test kit for HIV-2.

Employees will be tested in groups, and

no member of a group will receive test results until all results for the group are available. Employees will be notified by mail of negative results, and in person if the tests show evidence of HIV infection. If an employee's serologic status remains indeterminate after repeated testing, that person will be bled at more frequent intervals. Up to 5 weeks may transpire between testing and notification of results.

McKinney says his office took particular pains to maintain the confidentiality of employees. A file separate from other health information will be established for participants in the programme. Blood samples will be coded, and only the programme director and one deputy will be able to match the codes to the employees.

Safety officials at the Centers for Disease Control (CDC) in Atlanta are considering a testing programme for employees there, but a CDC spokesman says no decisions have been made on how or if such a programme will be implemented.

Joseph Palca



# Funding needed for private enterprise in genome sequencing

**Boston**

A LITTLE OVER a year ago, Harvard biologist Walter Gilbert surprised many colleagues and observers by resigning from a National Academy of Sciences advisory committee on the human genome project and announcing his intention to start a private company that would push ahead rapidly with both mapping and sequencing the human genome.

At that time, Gilbert's plans added fuel to a controversy over the appropriate goals and timing of the human genome project. Now, just as there appears to be a

growing consensus on the merits of launching the project in the near term, there also seems to be far less disagreement over the role of the private sector.

cost", providing that it is made publicly available. But he notes that in the course of the debate over the topic, many have under-estimated the size of the project. "With all the ballyhooing, it is important to remember that nobody has even sequenced *E. coli* yet," he says.

As a national genome project has slowly begun to move forward under the auspices of the National Institutes of Health (see *Nature* 332, 99; 1988), Gilbert acknowledges that his view of the role of his company — and of the private sector more generally — in the genome project has

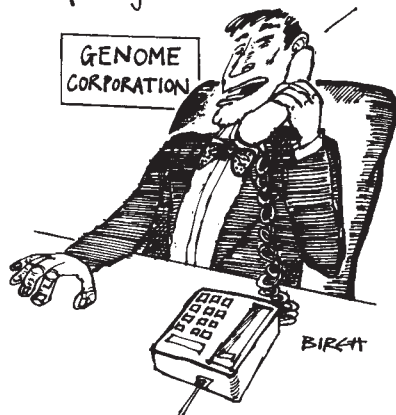
changed.

Gilbert now sees his venture as primarily a sequencing company that will try to interrelate with government efforts along the same lines. Charles Cantor of Columbia University, who sits on Genome Corporation's board, agrees there will be a need for contract work to complete the sequencing of the 3,000 million base pairs of the human genome.

Cantor suggests that once the goals of the genome project are clearly defined, companies might vie for contracts by performing sequencing work on a pilot scale and being judged according to their speed, cost and quality control. He expects that private companies will also have a role in the development of instrumentation.

**Seth Shulman**

... and for just \$10,000,  
you can have a gene named  
after you or a loved one...



growing consensus on the merits of launching the project in the near term, there also seems to be far less disagreement over the role of the private sector.

"While everyone else is fussing, I might as well go do it . . .", Gilbert, a Nobel laureate and former chairman of Biogen, told the press in February 1987. In the absence of any serious movement on the national level, his Genome Corporation, Gilbert said, would aim to complete a physical map of the human genome within a year of the company's start-up and to sequence major regions of the genome within three years thereafter.

One year later, Gilbert still seeks the funds to launch his private venture, although he remains hopeful that the company will materialize, perhaps as early as this summer. Gilbert says some of his financial problems result from the October 1987 crash of the stock market.

But as several scientists in the field noted, Gilbert's plans today are markedly different from his original outline. Eric Lander of the Whitehead Biomedical Institute at MIT sees Gilbert's plans as having been scaled down. Lander says he is in favour of "whoever can do the sequencing most efficiently at the lowest

## Genentech's boom is boosted by new clinical trial data

**Berkeley**

THE biotechnology company Genentech is riding high, thanks to new successes for its clot-dissolving drug, Activase (tissue plasminogen activator, TPA). On 26 March, favourable results were announced from two clinical trials of TPA; a placebo-controlled mortality study with heart-attack patients, and a trial comparing TPA to urokinase as a treatment for pulmonary embolism.

The heart attack study, involving more than 700 patients and conducted by the European Cooperative Study Group, showed a 51 per cent reduction of mortality at two weeks when patients were treated with TPA versus a placebo. The results lived up to Wall Street rumours that TPA would better streptokinase and Beecham Group's Eminase, a chemically modified form of streptokinase, both of which have shown mortality reductions of just under 50 per cent. Franz Van de Werf, director of the study, called the TPA results "the best in-hospital survival ever reported in a large-scale study of thrombolytic agents". This may help to justify the cost of Activase, at \$2,200 a dose, ten times the cost of streptokinase.

In the second study, conducted by Harvard Medical School, 45 pulmonary embolism patients were treated with either TPA or urokinase, the current treatment of choice for pulmonary embolism. The investigators found TPA to be superior for rapid clot dissolution and reduction of pulmonary artery pressure.

Genentech is expected to apply for FDA approval of TPA as a treatment for pulmonary embolism later this year.

In the first few months since its approval for treatment of heart attack patients, Activase has established a wide following in the United States. Stuart Weisbrod, an analyst with Prudential Bache Securities

in New York, says this is due in part to aggressive marketing, but also to data showing that the effectiveness of streptokinase drops sharply if the drug is not administered within an hour of the heart attack. Weisbrod recently asked 1,000 cardiologists their opinion of TPA. Of the 150 responses he has received so far, he said the doctors overwhelmingly chose TPA as safer, as it is often difficult to know exactly when a patient's heart attack occurred.

Analysts say Activase's US success is unlikely to be mirrored in Europe. Streptokinase has a strong medical following in Germany, and Eminase, already on the market in Europe, has better clot-specificity than streptokinase, and other advantages over TPA as well, such as a longer lifetime in the bloodstream and a lower price, at about \$1,000 per dose. In a study published in the 12 March issue of *The Lancet*, Eminase gave a 47 per cent reduction in 30-day mortality in heart attack patients. The drug is unlikely to reach the US market before 1990.

Sales of Activase have slowed from the \$58 million reported for the last 6 weeks of 1987 (see *Nature* 331, 202; 1988), but stock analysts say that was predictable, as hospitals complete the stocking of their shelves. Denise Gilbert, of Montgomery Securities in San Francisco, estimates January's sales at \$20 million, and February and March at \$11 million each. Most analysts predict 1988 sales to be between \$250 and \$350 million. Company officials will not predict 1988 sales, but point out that 2,000 patients a week are now being treated with TPA, twice the January treatment rate.

While basking in the success of Activase, Genentech has boosted its stock to a listing on the New York Stock Exchange.

**Marcia Barinaga**

# Canada makes plans for defence of its Arctic territories

Washington

CONCERN OVER Canada's plan to purchase a multi-billion dollar fleet of nuclear attack submarines from Britain or France was voiced in the US Senate Armed Services Committee last week. If the submarines come from Britain, permission will have to be sought for the transfer of US nuclear propulsion technology.

According to Senator James Exon (Democrat, Nebraska) the implications of the transfer have not been fully examined.

The new fleet's purpose is to defend Canadian and NATO sea lanes against Soviet submarines, and to keep Canada's Arctic Ocean free from hostile intruders. But the stimulus that triggered the decision was incursions by US, rather than Soviet, vessels into waters claimed by Canada.

In dispute between the United States and Canada is the famed Northwest Passage which connects the Pacific and the Atlantic through Amundsen Gulf, Prince of Wales Strait, Viscount Melville Sound and Lancaster Sound. Although the passage is only 20 miles wide where it winds between Banks Island and Victoria Island, both part of Canada's Northwest Territories, the United States regards the Northwest Passage as an international waterway over which Canada does not have jurisdiction. The US view is that accepting this claim would open the door to others, including that of Libya's

Canada's greatest concern is that it would be powerless if nuclear armed submarines used Canadian waters to pass between Atlantic or Pacific, or simply to hide out of sight under the Arctic ice. So far, there is no evidence that Soviet submarines, which might want to pass through Canadian waters to avoid the closely guarded seas around Iceland, have violated Canadian sovereignty. But US submarines seem to have done so. In 1986, three US attack submarines surfaced at the North Pole, and at least one of them must have passed among Canada's Arctic islands to get there.

Negotiations between the United States and Canada have failed to settle the issue satisfactorily. An Arctic Cooperation agreement was signed in January this year which pledged that US ice breakers would not again enter "waters claimed by Canada" without "the Consent of the Government of Canada". But the agreement conceded nothing on Canada's demands of sovereignty over the Northwest Passage.

The Canadian government's conclusion is that it must ensure that it has the means to patrol the Arctic waters. A plan to build the world's largest ice breaker, able to operate year round in the Arctic, has been announced. And to ensure that it can see under the ice, Canada wants a fleet of nuclear submarines. The choice is between Britain's Trafalgar class, which

are more expensive but better able to break through ice, and France's Rubis class. Both countries are pursuing the multi-billion dollar contract vigorously.

The United States supports Canada's increased commitment to defence spending, which has been one of the lowest in NATO, but it would prefer to

see the money go on conventional forces in Europe. And now, faced with the possibility that the first purchase order might be made as early as June, the US government is beginning to worry about the implications of a submarine sale.

If British submarines are ordered, then US technology will have to be employed. That will raise new questions of the transfer of highly secret technology. And there is, according to Senator Exon, the risk that even a minor accident in a US-powered Canadian submarine could result in US submarines being banned from foreign ports. But, although these matters

## French plans to revise doctorate

Paris

FACED with the greater internationalization of research and the dominance of English as the international *lingua franca*, at least in science, France decided, in 1984, to make its two-tier doctorate more like the English and US PhD. However, life in a country that pays little regard to titles, but rates scholarly pursuits highly, could not be that simple. In reforms which could become effective before the May presidential elections, science and higher education minister, Jacques Valade, is planning to revive some aspects of the previous system.

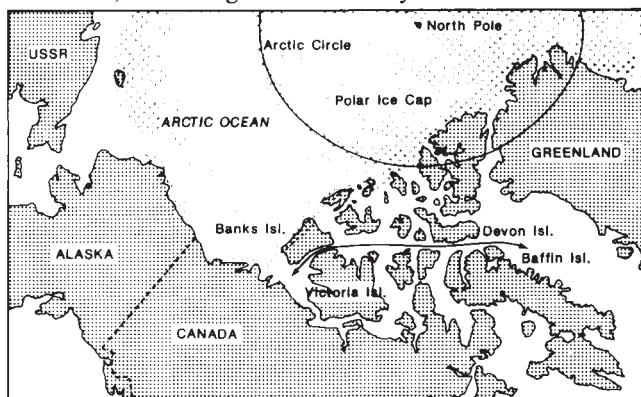
As is still the case in some European countries the French used to have two doctorates. The first, taken after the Diploma of Advanced Education (DEA), qualified the candidate to undertake independent research. In some disciplines, notably arts and humanities, a further period of directed research was necessary before a postdoc could supervise research or hold a senior university post.

The second doctorate — the Thèse d'Etat, or State Doctorate — was awarded after a long period of research under the paternal scrutiny of a supervisor and was seen by some as the culmination of a life's work. In 1984 the Thèse d'Etat was abandoned and the 'third cycle' doctorate upgraded to a PhD equivalent, based on three or four years' supervised research.

In 1986 Alain Devaquet, then research and higher education minister in the newly elected conservative government, proposed a series of university reforms, including the reintroduction of the Thèse d'Etat. This latter move was encouraged by old-guard university professors, close to senior cabinet ministers in the new government, who had seen their 'mandarin' status eroded by the more democratic PhD.

When Devaquet's proposed reforms produced student riots and his subsequent resignation, plans to return to a two-tier doctorate were put on the back burner. Now, under pressure once again from right-wing academics, Valade is planning to bring back the Thèse d'Etat, under a different name. Essentially, the post-doctoral qualifying period leading to admission to the academic community would involve further supervised research and, possibly, another thesis. The changes will initially apply only to arts and humanities; the science PhD will not be affected for the time being. Peter Coles

are sure to be considered, the final decision on a transfer of technology is bound to depend more on the political relationships between the two countries. Prime Minister Mulroney is due to visit US President Ronald Reagan in Washington this week. Alun Anderson



Colonel Gadhafi in the Gulf of Sidra.

The dispute was largely academic until August 1985. Then the US Coast Guard sent its massive icebreaker, *Polar Star*, from the Atlantic to Alaska through the Northwest Passage without asking the Canadian government for permission for the voyage. The United States simply informed Canadian authorities the trip would take place. That was sufficient to alert the Canadian government that as Prime Minister Brian Mulroney put it, they did not have "so much as a canoe" with which to assert their sovereignty over the Arctic territories.



## Act limiting nuclear liability heads for home strait

### Washington

THE Price-Anderson Act, the law that defines industry liability in the event of a nuclear power plant accident, is lurching towards renewal in Congress. Attempts to revive it before it expired last summer failed, as did those shortly afterwards. But now the Senate has passed and will shortly send to the House of Representatives its version of the new legislation, and observers from industry, environmental groups and congressional staff agree that final passage appears inevitable.

The Price-Anderson Act was created in 1954 to protect a fledgling nuclear industry from devastating liability claims in the event of a nuclear accident. The law created a pool into which all nuclear power plant operators would make contributions in the event of an accident. Liability limits crept up over the years, so that by the time the act expired last August utilities were required to carry \$160 million in insurance for each power plant they owned, and to be prepared to contribute \$5 million to the coverage pool. That amounted to a total industry liability of around \$700 million, far below even the most conservative damage estimates for any major accident.

The Senate version of Price-Anderson would raise the contribution to \$63 million per reactor, amounting to an industry-wide liability of more than \$7,000 million, a tenfold increase. Environmental lobbies have seen this as a tremendous victory, and the industry — aware that liability limits had been unreasonably low in the past — was happy to stave off unlimited liability that some had been pushing for.

Price-Anderson also covers Department of Energy contractors whose work

deals with fissionable materials. Without Price-Anderson, DoE contractors were threatening to refuse to sign contracts. But last year, the University of California agreed to continue to operate the DoE national laboratories at Livermore, Los Alamos and Berkeley under the terms of the War Powers Act, and a crisis was averted (see *Nature* 330, 103; 1987). The next major problem for DoE will be to find a contractor to run the weapons production reactor at Savannah River. DuPont has already told DoE it wants out, but now that Price-Anderson appears likely to gain approval, finding a replacement should be easier. Contractors are also covered up to about \$7,000 million in the event of an accident, but the government picks up the tab.

Environmental groups had sought to make contractors liable for damages if negligence contributed to an accident. But Price-Anderson will in essence retain its 'no-fault' approach, although the Senate agreed to permit DoE to assess penalties against contractors found negligent, in much the same way that the Nuclear Regulatory Commission may fine nuclear power plant operators.

An odd sticking-point may hold up final passage of the bill. In the Senate, an amendment was added that would extend Price-Anderson coverage to manufacturers of radiopharmaceuticals. That industry has had problems obtaining insurance, and some saw Price-Anderson as a convenient vehicle for providing that coverage. But the leadership in the House of Representatives does not agree, and a battle may be fought in conference between the two houses over this seemingly irrelevant point.

Joseph Palca

## France set to lead Europe?

### Paris

IT is paradoxical that the prospect of free trade in Europe from 1992 should stimulate a spirit of nationalism, with echoes of the late President de Gaulle. For this, superficially at least, was the impression conveyed at a morning conference on France's contribution to European science and technology held under the auspices of Prime Minister, Jacques Chirac, on 26 March. But, when electoral politics were put aside, leading researchers and technocrats provided an insight into practical problems confronting international research.

France is a leading supporter of several European 'big science' ventures, but, according to Chirac, needs to increase by 50 per cent its private-sector investment in technology research — equivalent to an extra FF25,000 million (£2,500 million) — in the next 7 years, in order to compete with West Germany. The way this has been translated in the government's research policy, however, gives little comfort to state-supported basic research. According to Chirac, "a dynamic policy supporting research and innovation is not achieved by voting budget increases for public sector research".

The range of government initiatives to "irrigate" the private sector with fertile young minds otherwise likely to dedicate themselves to basic research in the public sector is now familiar. The emphasis is firmly on tax incentives for research and development, industry-based grants for doctoral training and collaborative research between university and industry, based on 11 national research priorities (see *Nature* 329, 380; 1987).

While the politicians praised France's role in European research initiatives, those directly involved were more pragmatic. According to Philippe Lazar, president of the co-ordinating committee on medical research, "European cooperation works well in the laboratory, but hardly at all in the domain of communication and information exchange". Lazar wants to see more European science journals and better informed decision-makers. "Governments should be less concerned with financial returns and should devote more energy to multiplying opportunities for training and exchange at all levels, from researchers to administrators".

"Real research cooperation is easier to imagine than to achieve", says Lazar, who also feels that more effort should be spent on assuring compatibility of research procedures and evaluation of results, particularly in the field of public health, which requires "European-scale investment on a par with big science projects". Peter Coles

## Trees to benefit from Third World debt

### Washington

THE World Wildlife Fund (WWF) last week consummated a 'debt-for-nature' deal by purchasing \$1 million of Ecuadorean debt. The move follows an announcement the previous week of a similar arrangement with Costa Rica to the tune of \$3 million.

Purchasing discounted Latin American debt has suddenly become a popular way of encouraging conservation movements in developing countries, as it can frequently be purchased at a large discount. Last summer, Conservation International purchased \$650,000 in Bolivian debt for \$100,000 in exchange for a commitment to declare 3.7 million acres of tropical rain forest as a protected area.

Unlike the Bolivian debt swap, the WWF will give the note it purchased to a

non-government conservation organization in Ecuador, Fundacion Natura, which will exchange it through the Central Bank of Ecuador for bonds in local currency. Fundacion Natura will use the interest from the bonds to pay for continuing conservation projects. Bankers Trust Company and Citicorp/Citibank helped to arrange the debt purchase, which came to \$354,500.

The Costa Rican deal will be similar. Once the debt is purchased, it will be transferred to the Costa Rican National Park Foundation, which will also exchange it for local currency bonds.

Some of the bond proceeds may be used to purchase dry forest land for the proposed Guanacaste National Park.

Joseph Palca



# Szent-Györgyi and vitamin C

SIR—The book review by Walter Gratzer<sup>1</sup> describes the isolation of vitamin C in 1932. He says, correctly, that J. L. Svribely went from C. G. King's laboratories in Pittsburgh to Albert Szent-Györgyi's laboratory in Szeged, where Svribely tested Szent-Györgyi's hexuronic acid with guinea pigs and found that it was vitamin C. Reflecting the way events are recounted in the book, Gratzer then says that King, upon receiving a letter from Svribely with this information, "promptly and shamelessly jumped the gun and got a paper off to *Science*".

This is defamatory to King and is quite incorrect. It is compounded by Gratzer's statement that the book (by Moss) is "clear-eyed, scholarly and scrupulous". The pertinent events were as follows<sup>2,3</sup>: W. A. Waugh, a student in King's laboratory, obtained crystalline vitamin C from lemon juice, in September 1931, with constant assay activity in scorbutic guinea pigs. These results were repeated, and Waugh and King submitted an abstract for the 27–30 April 1932 meeting of the American Society of Biological Chemists. As nearly as I can find out, abstracts then had to be submitted before the end of February to be placed on the programme of the meeting. This is in accordance with King's statement that a few weeks after this submission, King received a letter from Svribely (as noted by Gratzer) in Szent-Györgyi's laboratory, saying that they were "just finishing their first assay in which animals . . . were protected from scurvy when given 1 mg of hexuronic acid daily" and that they were sending a report to *Nature* (published 16 April 1932)<sup>4</sup>. The report did not mention that Svribely had spent the previous year working with King and that he and King had co-authored a paper on preparation of vitamin C concentrates from lemon juice<sup>5</sup>. In short, the news was brought from Ghent to Aix, not, as Gratzer claims, from Aix to Ghent! In contrast to the omission of reference to their work by Svribely and Szent-Györgyi, King and Waugh in *Science*<sup>6</sup> stated that recrystallized vitamin C from lemon juice "is apparently identical with the hexuronic acid described by Szent-Györgyi", thus giving credit to Szent-Györgyi for his role.

Far from "jumping the gun", King actually *delayed* submission of his manuscript to *Science* until he had checked the spurious claim of O. Rygh that vitamin C was methylornarcotine<sup>3,7,8</sup>.

It should be noted that S. S. Zilva had reported erroneously that Szent-Györgyi's hexuronic acid was not Vitamin C<sup>3,7,9</sup>, and that King and Waugh corrected Zilva's mistake<sup>1</sup>.

This brings up the question: when is a vitamin identified? The answer is: not until its biological activity is established. Funk, who had predicted the existence of

an anti-pellagra 'vitamine' (he coined the word 'vitamine', now 'vitamin'), isolated nicotinic acid from yeast in 1913<sup>10</sup>, but it was not tested against canine black tongue or human pellagra until 1937 and so was not recognized as a vitamin until then, without credit going to Funk. Szent-Györgyi isolated hexuronic acid in 1928<sup>11</sup>, but it was not tested against scurvy until 1932. The test was carried out by Svribely, not by Szent-Györgyi<sup>12</sup>.

At my invitation, in 1979, King described the history of the isolation of vitamin C at the annual meeting of the American Institute of Nutrition<sup>1</sup>. King's scientific conduct in the vitamin C episode, as on all other occasions, was exemplary and punctilious<sup>2</sup>, and, in my opinion, over-reticent on this issue, despite the fact that he did not share in the Nobel Prizes for vitamin C in 1937. It is unfortunate that this slur has appeared within a short time of King's death on 23 January 1988.

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SIR—I would like to correct some misconceptions appearing in Walter Gratzer's otherwise excellent review of *Free Radical: Albert Szent-Györgyi and the Battle Over Vitamin C* (*Nature* **331**, 397–398; 1988). I had the privilege of collaborating closely with Szent-Györgyi during the last 12 years of his life and enjoyed the friendship of many of those who collaborated with him before and during that time. To state, as the reviewer does, that Szent-Györgyi "often perverted" the talents of his scientific followers is a concept that does not come out of the book and incorrectly reflects the integrity and spirit of the scientific collaborations which occurred.

The reviewer also states that the National Foundation for Cancer Research which supported Szent-Györgyi's work for 13 years "collapsed in squalor and recrimination" and yet the book (p. 257) describes how "for reasons difficult to fathom" the foundation's fund-raising fell but "has since begun to stabilize". I currently receive funding from the foundation and my involvement with the "international, interdisciplinary, laboratory

without walls" approach of its support for basic research remains one of my most exciting experiences. It is true that in the last year of his life Szent-Györgyi was in conflict with the foundation's administration, but I believe the previous 12 years of mutual goodwill and support far outweighed this unfortunate situation.

Finally, and here the reviewer does correctly reflect the book, the image is given that "influenced by his young wife, Szent-Györgyi became alienated from his family and friends" and at the end became "lonely, embittered and unfulfilled". During that last summer (and for many before that) my family and I were house guests of the Szent-Györgyis in the summer cottage by their house. Marcia Szent-Györgyi lovingly devoted herself to the care of her husband and encouraged visitors whenever possible (one such visitor was Linus Pauling). Although Szent-Györgyi was seriously ill, he remained interested in and positive about the science in his laboratory, and he retained his impish sense of humour and philosophical view of life. If there is one criticism I would make of the biography of Szent-Györgyi, it would be that the author did not allow himself enough time to obtain a wider and more balanced view of those last few years.

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## India and China

SIR—In my opinion the letter by N. H. Antia, "India since independence" (*Nature* **331**, 384; 1988) contained inaccurate statistics and factual errors.

It is difficult to compare India and China because precise statistics are difficult to obtain from a closed system such as China's. Nevertheless, available data indicate that India leads China in most fields, besides having the third largest technical manpower in the world (after the Soviet Union and the United States).

China lost an entire generation of scientists to the cultural revolution while science in India benefits from free interaction of Indian scientists with those in Western and Eastern bloc countries. The number of contributions to scientific journals coming from Indian as opposed to Chinese laboratories demonstrates this.

Moreover, the sound defeat of the Chinese during the 1979 Sino-Vietnamese conflict indicated the backwardness of the Chinese arms industry, which may reflect the state of her heavy industry as a whole.

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# How not to catch attention

*Scientists as authors seem to reject the convention followed by writers in other fields that the first sentence is important. Why are platitudes preferred?*

CONVENTIONS of calculated banality seem to be spreading through the scientific literature. That, at least, is the impression outsiders would be given by even the most casual reading of the first sentences of the articles in almost any contemporary journal. What seems to have happened is that authors have decided that the only seemly beginning to an account of their investigations is a platitude that does not even accurately signal the direction in which readers' attention should be diverted, and which conveys no sense of the excitement that may follow.

This convention flies directly in the face of that followed in other forms of literature, where there seems to be a general understanding that the first sentence of any piece of prose has the crucial function of capturing readers' attention.

Sometimes, authors do this by almost theatrical means, as with the sentence "Hell!, said the duchess" beginning Eric Linklater's story *Sealskin Trousers*. Sometimes they are philosophically thought-provoking, as with Tolstoy's sentence "Happy families are all alike; each unhappy family is unhappy in its own way" in *Anna Karenina*. The ideal is that a first sentence should be arresting enough to sustain readers' imaginations through whatever lies ahead.

The conventions of journalism go further. The first sentence is supposed not merely to catch attention, but to encapsulate the essence of what follows. So it is common to find first sentences such as "Sudden summit mania has gripped the White House" (in a British newspaper last weekend) or "Mr Gavin Laird (a British trade union leader) is a real smoothie" (the same source). These quotations incidentally conform with the extra rule once promulgated by the Associated Press that a first sentence should not be longer than fourteen words. Although critics may complain that these same quotations also illustrate how the conventions of journalism encourage exaggeration, there can be no doubt of their effectiveness.

So why do scientific authors lean in the opposite direction? Thus

Recently, interest in effects that can be ascribed to the non-linear behaviour of plasma waves and their influence on the containment and heating of a laboratory plasma, has grown.

or

Much attention has been focused in recent years on the behaviour of weakly non-linear waves in dispersive media and considerable

interest has centred on those waves that propagate in the form of localized pulses known as solitons.

Who would guess that the first quotation introduces an article claiming that sufficiently intense laser pulses may be self-focusing when travelling through a plasma and that the second is a calculation of the conditions under which solitons will be set travelling through plasmas by the interaction of three pulsed laser beams, both questions of some importance to those working with experimental thermonuclear machines? (To avoid bias, these and later first sentences are taken in order from successive articles in a single issue of a European journal, which is not identified because its practice is not noticeably worse than that of others.)

One justification of these banalities might be that they include key-words pointing to the content of what follows, but that is plainly a lame excuse. For then, both these first sentences would have said something about the context in which "interest" and "much attention" have, respectively, been evoked. The same is true of the sentence

Zinc oxide is one of the metal-oxide types of material with important physical properties for both basic studies and applications.

That, surprisingly, introduces an experimental and theoretical study of the influence of departures from stoichiometry on the electron band structure of metal oxides which cannot but be relevant to the understanding of the now-fashionable superconducting oxides.

Another possible explanation of vacuous first sentences is that they are meant to warn potential readers away from material that they might find uncomfortably difficult to follow. But do not authors lucky enough to find themselves in print have a duty to capture the interest of as many readers as possible? Neither of the sentences introducing the two plasma physics articles warns readers that the texts that follow are strictly mathematical, for example, while there is no reason to suppose the authors of the zinc oxide article do not wish for readers.

Diffidence of a kind is nevertheless part of the calculation of the authors of these platitudes, as shown by the sentence

Processes that are inhibited by the presence of a potential well are ubiquitous in many areas of natural sciences, ranging from nuclear physics over low-temperature solid-state physics and chemical reactions up to biological processes.

Again, the real objective of the article (a discussion of the interesting question of how the quantum-mechanical tunnelling of a system from a metastable to a stable state is to be treated when the temperature is other than absolute zero) is well-concealed; reaction rates are mentioned almost in passing, as if the two authors had been modestly persuaded that they could not expect people to read their article without saying that the canvas stretches from nuclear physics to biology.

By contrast, the much more direct

The basic idea of Daydov's soliton theory is that vibrational energy becomes self-trapped through interaction with low frequency phonons.

seems deliberately intended as a warning to those unfortunates not instantly familiar with Davydov's theory of solitons (a Soviet researcher's calculation of mechanical vibrations in macromolecules) that they had better turn the pages in search of something simpler. That betokens not so much diffidence as the opposite.

Simpler platitudes are nevertheless much more common, as in

High-temperature superconductivity occurs in quaternary materials of the III-II-Cu-O system, where II denotes an alkaline-earth element, and III a rare earth.

The article that follows suggests a way in which electron coupling in mixed oxides may be sustained by the exchange of "plasmons". The function of the opening sentence seems to be to ground what is necessarily a somewhat conjectural piece in a sentence with which nobody will quarrel. It is sheer bad luck that the bismuth and thallium superconducting oxides came on the scene after the article was accepted for publication last October. The authors (three) would not doubt say that, because their first sentence had no particular purpose, the fact that it has been proved false is immaterial.

Up to a point, there is no particular lesson to be learned from this catalogue of banality unless it is that researchers are less conscious than other professional people of the need to capture readers' attention. The now-common convention on first sentences is hardly the most glaring complaint against the literature of science. But its wide adoption may fairly be taken by outsiders as proof that the authors of scientific articles, by turning their backs on a convention universally followed by others, do not much care how easily their work can be read. **John Maddox**



## Wildlife protection

## Control of feline delinquency

Robert M. May

As I write, I am watching the neighbour's cat in the bottom of my garden, which appears to be trying to repeat its recent accomplishment of killing a young squirrel. Just how much mortality is inflicted on wildlife by the largely 'recreational' hunting of domestic cats? Churcher and Lawton provide some answers from their recent study<sup>1</sup> of around 70 domestic cats in the village of Felmersham in Bedfordshire, United Kingdom.

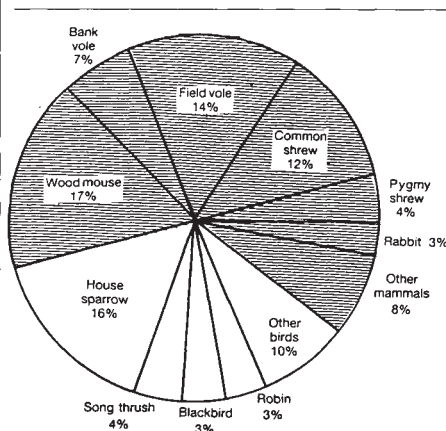
There are 173 houses in the nucleus of Felmersham village, including one working farm. These provided 78 cats that Churcher and Lawton studied at some time over a single year; at any one time, however, there were only about 70 cats in the study. This incidence of cats is roughly twice the British average, which is around one cat for every four houses<sup>2</sup>. Churcher and Lawton based their study on the prey items brought home by the cats. Although this method clearly provides only a lower limit to the predatory activities of the cats, it is not easy to do better. The two main alternatives are systematically to examine faeces (which is extremely labour-intensive, and can be difficult to translate into number of prey items) or to look at the gut contents of cats killed on the road or as a result of pest-control programmes (which can give a biased picture and would be difficult to translate into predation rates)<sup>1</sup>.

Churcher and Lawton counted a total of 1,090 prey items brought home during the year: 535 mammals, 297 birds and 258 other unidentified animals, an average of around 14 items per cat per year. Among the prey were 22 species of birds and 15 of mammals, the most common being woodmice (17% of the total), house sparrows (16%) and field voles (14%). These findings are summarized in the figure.

Overall, birds comprise 35% of the identified total catch. Churcher and Lawton note that "this is lower than folklore would suggest but higher than the results obtained in most research programmes". In the only previous large-scale study<sup>3</sup> of house cats, in various locations throughout Britain, Howes found that birds form about 21% of the catch (and mammals most of the rest). Fitzgerald and Karl reviewed<sup>4</sup> studies on feral cats in Europe and Australia, and concluded that songbirds usually form less than 7% (but by volume, not number, which impedes comparison) of cats' diet in agricultural habitats and natural grasslands. But McMurtry and Sperry find<sup>5</sup> that the proportion of birds in the diet of feral

cats in Oklahoma increases by a factor of 4 as one moves from rural locations to residential and roadside areas, so that Churcher and Lawton's findings are not inconsistent with the results summarized by Fitzgerald and Karl.

Examining their data in more detail, Churcher and Lawton show that hunting success is correlated with weather. Although temperature alone seems to



Composition, by species, of the 832 identified prey items (identified from a total of 1,090) brought home in one year by around 70 cats in a Bedfordshire village (after ref. 1.)

have no influence, significantly fewer prey items are brought home in winter than in other seasons. Not surprisingly, these domestic cats also appear to hunt less on wet or windy days. For both sexes, young cats catch more prey than old cats. Hunting patterns also depend on the location of the cat's house within the village. Cats in central areas of the village catch proportionately more birds than cats on the village edge. Female 'edge' cats also bring home more prey items than female 'core' cats, although male cats manifest no such difference.

Churcher and Lawton selected house sparrows as the easiest species for assessing the impact of cats. Their survey of this species was helped by the absence of tree sparrows and by the "colonial habit, garrulity and quarrelsomeness of house sparrows [which] makes them easy to find in the relative calm of early morning". They find that the 130 house sparrows brought in by cats over the course of the year represented one-third of all sparrows in the village at the start of the breeding season. Noting that the actual number killed may be significantly higher if not all prey is brought home and found by owners, Churcher and Lawton conclude that at least 30% of sparrow deaths are

caused by cats. The extent to which this figure can be extrapolated to other bird species is uncertain.

Churcher and Lawton give many caveats. For one thing, it is unlikely that cats are so obliging as to bring home all the animals they kill. When studying well-fed cats on a farm in Illinois, George found<sup>6</sup> that about 50% of prey were indeed brought home, with the other 50% being eaten, scavenged by other animals or simply not found. Thus, results obtained by assessing items brought home may be reliable as to species composition (although even here one may have reservations), but not as to absolute numbers. Given McMurtry and Sperry's observation<sup>5</sup> of a fourfold variation in the proportion of birds in the diet according to the environment of the cat, one must wonder about the extent to which results for a small rural village extend to more urban regions. Another thing I would like to know more about is whether the depredations of belled cats are significantly less than those of unbelled animals.

It has been estimated<sup>7</sup> there are around 6 million domestic cats in Britain. At an average of 14 prey items per cat, this adds up to about 100 million birds and small mammals killed each year by well-fed domestic cats. One way of thinking about this is to ask whether the number of birds, small mammals and other creatures killed each year by carnivorous domestic pets exceeds the corresponding number killed by native carnivores (many of which are now extinct) in Britain before the advent of humans.

This is an exceedingly difficult question to answer. On the one hand, today's 'standing crop' of cats is sustained mainly by pet food and is undoubtedly larger than could be maintained purely by a dynamic interaction with native prey. On the other hand, it could well be that the interactions were such that both prey and predator populations were lower in the pristine situation, so that not only could fewer small mammals and birds have been killed each year, but there could simply have been fewer animals in the first place. Add to this the profound changes in landscape patterns associated with human activities, and comparisons between the demography of species 10,000 years or more ago and today become very uncertain.

Another way of thinking about predation on wildlife by domestic cats is to ask why it elicits so little public concern, in contrast with the sometimes passionate feelings expressed about laboratory animals. Thus it is necessary — and properly so, in my opinion — to observe rigorous protocols and review procedures before undertaking any experiment in which live prey are fed to cats to study predatory behaviour, and the Home Office has been admirably diligent in prosecuting any violations of these

procedures. It seems to me, however, somewhat inconsistent to be so properly careful in overseeing the killing (or "sacrifice", in the bizarre euphemism of the trade) of a few laboratory-bred birds in pursuit of understanding how the world around us works, but not to object to the fall of about 10 million sparrows every year as sport for well-fed household cats.

But perhaps it is sensible to stick to regulating those activities that lend themselves to regulation, and to leave the rest of the world alone. As Adlai Stevenson once wrote, when vetoing proposed legislation while he was Governor of Illinois, "...The problem of the cat vs. the bird is as old as time. If we attempt to resolve it by legislation, who knows but

what we may be called upon to take sides as well in the age-old problems of dog vs. cat, bird vs. bird, or even bird vs. worm. In my opinion, the State of Illinois and its local governing bodies already have enough to do without trying to control feline delinquency". □

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## Physical chemistry

# Self-assembling surfaces

R. J. P. Williams

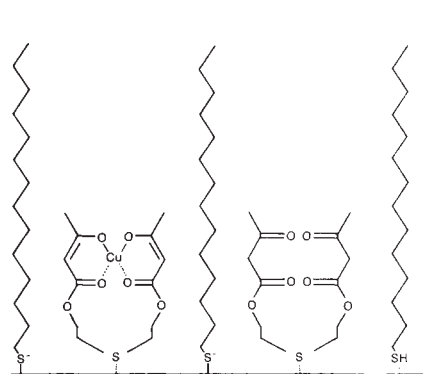
CHEMISTS have been trying for many years to produce self-assembling monolayers, similar to those of natural membranes but deposited on electrodes to give a selective response to solutes. I. Rubinstein *et al.* report on page 426 of this issue that they have achieved this objective by designing a monolayer surface-covering for a gold electrode which responds selectively to copper ions in solution. Apart from the technical achievement, there are three points of great interest: the way in which chemical contact is made with the electrode; how the monolayer assembles; and the selectivity of the reagents chosen for the surface layer.

In general, the greatest electroactivity is obtained with monolayers of molecules that bind to the electrode by an unsaturated nitrogen atom or a saturated sulphur atom, the latter being preferred. For example, molecules with either binding atom have been used to perform reversible electrochemical redox reactions on proteins such as cytochrome-c at a gold electrode (Frew, J.E. & Hill, H.A.O. *Phil. Trans. R. Soc.* **B316**, 95-106; 1987). Usually, reversible reactions are not possible with uncoated metal electrodes. The coating of the electrode has the secondary function of preventing the irreversible absorption of the substrate onto the electrode. Monolayers have been made with the selectivity of the antibody-antigen reaction, and biosensors have been developed (Ngo, T.T. (ed.) *Electrochemical Sensors in Immunological Analysis*, Plenum, New York, 1987). These do not, however, self-assemble.

It might seem that all that is required is a single reagent which binds both to the electrode and selectively to a molecule in solution. Curiously, Rubinstein *et al.* find

that this is not so. Their monolayer is made from an equimolar solution of 2,2'-thiobisethyl acetoacetate (TBEA) and *n*-octadecyl mercaptan (OM). This monolayer seems to be structured, so the authors rightly call it self-assembled. But it is not clear why the monolayer is structured and is not just a random two-dimensional liquid on the gold surface. It could be that surface repulsion between

The self-assembled monolayers of Rubinstein *et al.* The 'chelating' reagent TBEA (which binds to ions in solution) alternates with the long-chain thiolate, OM, shown in both ionized and unionized states. Such stacking also creates potential binding sites for copper atoms near the surface, which involve sulphur atoms. One of the enolate centres (shown bound to the copper) and perhaps the gold surface, could also be involved. Selectivity for copper ions could arise from their preference for a tetrahedral binding geometry not favoured by iron. The same steric principle is exploited in the use of dissolved 2,9-dimethyl orthophenanthroline as a selective copper reagent or indeed in the 'blue' sites of copper proteins such as plastocyanin.



thiolate (OM) molecules is such that the TBEA, as well as binding to the gold, is acting as an ordered diluent, even hydrogen-bonding in its enolate form to the thiolate (see figure).

Ordering in biological membranes depends on such properties as charge-matching, hydrogen-bonding and spatial fitting, as with the TBEA/OM monolayer. But there is much evidence that components of a biological bilayer interact with the substratum of proteins and even with the cytoskeleton. Such interactions are locally selective which seems unlikely for a featureless gold surface. Crystallization can also be called self-assembly, but

biological self-assembly may not involve the principle of cooperative repetition as is found in salt crystals.

Apart from the interest in developing organized electroactive surfaces, the mechanism of chemical recognition is also intriguing. Here, the work of Rubinstein *et al.* is difficult to understand. Why ligands are selective for copper over iron is an old analytical problem. In neutral solutions ( $pH=7$ ), where hydrolysis is not a severe problem for Cu(II) and Fe(II) ions, many selective reagents are known because Cu(II) binds more strongly to virtually all ligands (the Irving-Williams series). Selectivity is poor with oxygen donors and best with sulphur-donor ligands. The surprise in the paper of Rubinstein *et al.* is that a tetradentate (having four binding groups) oxygen donor (enolate) should give such selectivity. The authors suggest that this is because of geometric discrimination, but this does not really seem reasonable because, unlike the designed ligands of Lehn, Pedersen and Cram (who won the 1987 Nobel prize for chemistry), the framework is not rigid.

It is also conventional wisdom that  $VO^{2+}$  has the same stereochemical preference for ligands as Cu(II), but Rubinstein *et al.* find that its electrochemical behaviour at the treated surfaces is very different. The explanation given by Rubinstein *et al.* is that there could be an initial reduction of a small amount of copper directly on the gold surface. Does the selectivity

rest in interactions of copper atoms with the sulphur of the monolayer, which would not arise so readily with iron or vanadyl because they cannot be reduced easily to neutral atoms? Selectivity in such a heterogeneous surface may be rather different from that in solution because of packing arrangements in the construction associated with the monolayer. In this case, self-assembly could be quite sophisticated. It seems that neither the self-assembly, nor the electrodic processes are yet understood. □

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## Genetics

## Driving genes and chromosomes

Brian Charlesworth

THERE are several genetic and chromosomal systems in which Mendel's first law — the equal probability of transmission of maternal and paternal alternative alleles or homologues — is violated. This phenomenon was named 'meiotic drive' in 1957 by Sandler and Novitski, who drew attention to the fact that it operates as an evolutionary force which can cause an increase in the population frequency of the allele or chromosome which is favoured in transmission, even if it confers a disadvantage on its carriers in terms of fitness at the level of the individual. It is the best-studied case of the conflict of interest between a piece of selfish DNA, which attempts to increase its representation in the gametes transmitted to the next generation, and the individuals that host it within their genomes. The phenomenon of meiotic drive provides an opportunity for studying how unequal transmission is accomplished, how it is controlled genetically and what forces affect the frequencies of driving elements within natural populations, as well as the relationship between these questions. Developments in all three areas were described at a recent meeting\*.

Progress in the genetic and molecular analysis of meiotic drive has been most rapid in the case of the segregation-distortion system of the fruitfly *Drosophila melanogaster*. This involves a gene complex (*SD*) near the centromere of the second chromosome, which causes the sperm of males heterozygous for *SD* to fail to develop normally if they carry the non-*SD* homologue. *SD* is present at low but stable frequencies in most wild populations. There seem to be three main loci contributing to the genetic difference between *SD* and the normal chromosomes *Sd*, *E(SD)* and *Rsp* (B. Ganetzky and R.G. Temin, University of Wisconsin, Madison). The product of the distorter gene *Sd* apparently interacts with sensitive alleles (*Rsp*<sup>s</sup>) at the responder locus *Rsp*, carried by non-*SD* second chromosomes, causing *Rsp*<sup>s</sup> sperm to develop abnormally. *SD* chromosomes (and many naturally occurring non-*SD* chromosomes) carry an insensitive form of *Rsp*, *Rsp*<sup>i</sup>, so that they are protected from destruction by the *Sd* product. They also carry an allele,

*E(SD)*, at a third locus that considerably strengthens the degree of distortion by *Sd*, and can itself cause distortion of segregation at the *Rsp* locus when present in double dose. The three loci are closely linked, and recombination between *SD* and normal chromosomes is usually prevented by the presence of chromosomal inversions associated with *SD*.

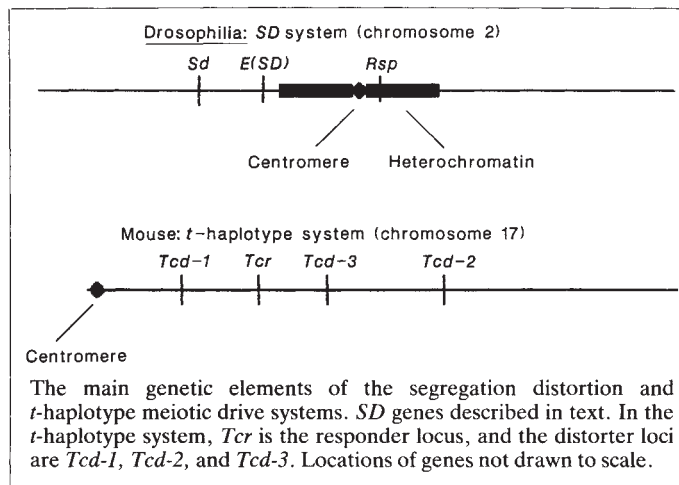
*Sd* differs from wild-type and from deletion mutants lacking *Sd* activity by the presence of a 5-kilobase duplication of part of a locus in salivary chromosome

genetic basis, with several closely linked genes involved in causing distortion of segregation in favour of the *t* haplotype (M.F. Lyon, MRC Radiobiology Unit, Chilton). There are at least three distorter loci and one responder locus (see figure). Molecular cloning of parts of the *t* region has been accomplished, and it is clear that *t* chromosomes differ from normal chromosomes by at least two large inversions that suppress crossing over in heterozygotes for *t* haplotypes. A gene (*tctex-1*) has been cloned that apparently causes male sterility of the same type as that seen in viable homozygotes for *t* haplotypes (D. Bennett, University of Texas, Austin). This may turn out to be a distorter locus, as these have not been separated genetically from the *t*-region sterility genes.

The population and evolutionary dynamics of meiotic-drive systems have been intensively studied theoretically (M.W. Feldman, Stanford University; my own work), and provide explanations for the general features of systems such as *SD* and *t*. The multi-locus nature of the differences between normal and distorting chromosomes that are required to produce effective drive imposes stringent conditions on the linkage relations between the distorter and the responder loci, if these are to experience an evolutionary advantage.

Furthermore, there is selection for factors that reduce the frequency of crossing over between these loci, and between them and modifiers of the intensity of drive. This accounts for the associations between the drive factors and inversions in both systems. We are at present far from having a detailed knowledge of the numerous parameters needed for detailed predictions of the behaviour of populations (A. Beckenbach, University of British Columbia, Vancouver; J. Curt-singer, University of Minnesota, Minneapolis). The use of molecular markers to characterize genotypes at the meiotic-drive loci, instead of the tedious genetic tests needed up till now, will revolutionize future population studies.

B chromosomes are additions to the normal complement of chromosomes that are not required for successful development and reproduction, and indeed often adversely affect survival or fertility. They exhibit various patterns of differential transmission to the gametes, often involving unequal segregation at mitosis rather than meiosis, and are present at variable numbers in natural populations (R.N. Jones, University of Wales, Aberystwyth). An unexpected property of a B chromosome, which may be the ultimate



band 37D2, the known location of *Sd* (P.A. Powers, University of Wisconsin, Madison). It is thus almost certainly the primary genetic lesion in *Sd*. Genetic experiments (T.W. Lyttle, University of Hawaii) and cytological observations (S. Pimpinelli, University of Bari, Italy) show that *Rsp* is a duplicated sequence, with larger copy numbers present in *Rsp*<sup>s</sup> than in *Rsp*<sup>i</sup> chromosomes.

Cloning and sequencing of *Rsp* has shown that it is an AT-rich, 120-base-pair repeated sequence (C.-I. Wu, University of Rochester). *Rsp*<sup>s</sup> chromosomes from the wild contain only 25 copies on average, whereas a typical *Rsp*<sup>i</sup> chromosome contains 700 copies. There is a correlation between the degree of sensitivity of naturally derived chromosomes and their copy number. There is little doubt that the repeated sequence is the target for the product of *Sd*. The successful cloning of the distorter and responder loci provides hope that a detailed understanding of the mechanism of drive will soon be obtained.

Genetic analysis of the *t*-haplotype system of mice, where males heterozygous for wild-type and *t* haplotypes produce functional sperm that are largely *t* in genotype, shows that it also has a complex

\*Genetics and evolutionary biology of meiotic drive University of Hawaii, Honolulu, 6-8 January 1988. In honour of the late Larry Sandler (University of Washington, Seattle).

in selfish behaviour by a piece of DNA, has recently been discovered in the parasitoid wasp *Nasonia vitripennis* (J.H. Werren, University of Rochester). As in other Hymenoptera, the sex-determination system in this species involves the production of diploid females and haploid males, from fertilized and unfertilized eggs, respectively. A paternally transmitted B chromosome present in some individuals from natural populations causes the elimination of the paternal genome from

fertilized eggs, so that they develop as haploid males with a maternally derived genome, apart from the B chromosome itself. Theoretical analysis shows that this chromosome experiences a transmission advantage in populations where the sex ratio is female-biased, as is normally the case in *Nasonia*. □

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## Vascular biology

# Put out to contract

John Gordon

THE role of the endothelium in maintaining vascular homeostasis now has another layer of complexity, with the discovery, reported on page 411 of this issue<sup>1</sup> by Tomoh Masaki's team, of a novel vasoconstrictor produced by endothelial cells. The paper describes the characterization of endothelin, which contracts vascular strips with greater potency than any known mammalian vasoconstrictor and is secreted by endothelial cells in response to various stimuli. (Now reread the title, with the emphasis on the last syllable.)

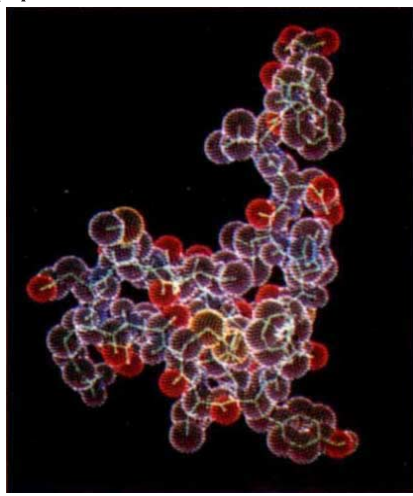
This discovery is the latest in a long series that attempts to elucidate endothelial function. Not much more than a decade ago, the vascular endothelium was regarded as a sort of nucleated dialysis bag that lines the blood vessels, keeps most of the plasma proteins from escaping, and presents a haemocompatible surface to stop blood from clotting. Otherwise, it was believed to take little active part in any vascular proceedings. In the past few years, however, the number of activities in which the vascular endothelial cells are known to engage has become almost embarrassingly large, including blood coagulation, fibrinolysis, platelet function, granulocyte traffic, lymphocyte recirculation, antigen presentation and smooth muscle proliferation, as well as the control of vascular tone.

The realization that endothelium is involved in vasoregulation first came with the discovery<sup>2</sup> of prostacyclin, which is produced by endothelial cells (among others). Prostacyclin, a vasodilator in many vascular beds, can act like a local hormone to increase blood flow in response to physical or chemical stimuli. Endothelial cells also secrete a range of other vasoactive arachidonate metabolites.

The next revelation about the contribution of endothelium to vasoregulation was Furchgott's discovery<sup>3</sup> of the activity called endothelium derived relaxing factor (EDRF). The report in *Nature* last year<sup>4</sup>, which showed that nitric oxide is equivalent to EDRF (at least, to one

EDRF) was accompanied by a News and Views article by Paul Vanhoutte<sup>5</sup>. Vanhoutte's group, among others, had previously reported that endothelium-dependent vascular contractions are induced by several stimuli, including hypoxia<sup>6,7</sup>, and further work established that a peptide from endothelium produces sustained vasoconstriction<sup>8,9</sup>.

Although endothelin seems to be this peptide mediator, some oddities remain



Putative three-dimensional structure of the endothelium-derived potent vasoconstrictor peptide, endothelin. The structure was estimated by free-energy minimization from the determined disulphide-bond topology and primary structure using the program MODEST. The amino-terminal half is convoluted into a rigid loop by two intrachain disulphide linkages (yellow). (Courtesy of M. Yanagisawa et al.)

to be resolved: the calcium ionophore A23187 has been reported to be stimulatory and inhibitory<sup>10</sup>, and the endothelium-dependent contractions induced by hypoxia are kinetically quite different from those mediated by endothelin. As produced by porcine aortic endothelial cells in culture, endothelin is a 21-amino-acid peptide with free amino- and carboxy-termini, and has a relative molecular mass of 2,492. It has four cysteine residues forming two sets of intrachain

disulphide bonds (see figure). Although not previously found among mammalian peptides, this general structure is common to a group of peptide toxins of non-mammalian origin that act on ion channels in cell membranes.

Sequence evaluation<sup>1</sup> shows that endothelin is similar to  $\alpha$ -scorpion toxins, which bind to tetrodotoxin-sensitive Na<sup>+</sup> channels and thus block neuronal transmission. Masaki and colleagues propose<sup>1</sup> that endothelin acts directly on membrane ion channels, citing as evidence their observations that the contraction of vascular smooth muscle induced by endothelin is dependent on extracellular calcium ions, and that the action of endothelin can be inhibited by calcium-channel blockers such as nicardipine. However, although these observations are consistent with the idea that endothelin is an endogenous agonist for dihydropyridine-sensitive voltage-dependent Ca<sup>2+</sup> channels in the plasma membrane of smooth muscle cells, it remains equally possible that the activation of calcium channels is secondary to stimulation of a receptor.

Endothelial cells were found<sup>1</sup> to contain messenger (m) RNA encoding the prepro-form (precursor) of endothelin, and the deduced amino-acid sequence for prepro-endothelin reveals a eukaryotic translation initiation site followed by a characteristic secretory signal sequence, suggesting that the peptide is synthesized and processed similarly to many peptide hormones. However, although paired basic amino-acid residues recognized by processing endopeptidases are found at Lys 51–Arg 52, the next dibasic pair is not found until Arg 92–Arg 93, suggesting that the mature peptide is generated by unusual proteolytic processing between Trp 73–Val 74 through an endopeptidase with specificity similar to chymotrypsin. The nature, intracellular location and regulation of this 'endothelin-converting enzyme' are all topics that are worth future study.

Endothelin is produced constitutively by pig aortic endothelial cells in culture, although Masaki's group<sup>1</sup> recognizes that endothelial cells in culture could share some features of 'damaged' endothelium *in vivo*; this is particularly likely under the conditions used in these experiments, in which the cells were kept for many days without serum. The mRNA encoding the prepro-peptide increases rapidly when the cells are exposed to thrombin, adrenaline or the calcium ionophore A23187, all of which are known to induce the release of a vasoconstrictor activity from endothelium (and, in other experiments, to induce the release of EDRF). The mRNA encoding prepro-endothelin is found in porcine aortic intima *in situ*, but not in porcine brain, lung or kidney, suggesting that the endothelium of the microcirculation in these tissues does not normally produce



substantial quantities of endothelin. In endothelial cells cultured under flowing conditions, the prepro-endothelin mRNA is substantially reduced (gene expression is down-regulated). If this observation can be extrapolated to the situation *in vivo*, the rate of blood flow may regulate the balance between the production of endothelin and EDRF, which could explain the phenomenon of flow-induced endothelium-dependent vasodilation<sup>10</sup>. The precise mechanisms that control this process remain to be elucidated, but it is known that endothelial cells possess mechanosensors which respond to changes in flow by regulating the activity of ion channels (for further discussion, see Lansman's recent News and Views article<sup>11</sup>).

Regional differences in the production of endothelin would be an important factor in determining its pathophysiological significance, as would a better understanding of the factors that regulate its production, either on a long-term basis (for example, local conditions of gas tension and blood flow) or transiently (such as changes in the concentrations of local hormones). Further, the distribution of receptors and/or ion channels for endothelin is not known: contractions have been observed in mesenteric, femoral, renal, pulmonary, coronary and basilar arteries from various species, and increases in pressure have been seen following injections into the perfused coronary (but not pulmonary) bed, as well as after intravenous injection into rats.

Analysis of the pathological role of endothelin (for example, in hypertension or in vasospasm) leads to a consideration of pharmacological intervention with a view to therapy either by receptor antagonism or by interfering with intracellular transduction mechanisms involved in its secretion. At this stage, very little is known about either. However, manipulating the secretion or action of endothelin would affect only one component of endothelium-dependent vasoregulation, and the balance between production of the peptide and of vasodilators (whether from endothelium or from elsewhere) could profoundly affect local vasoregulation, as could the kinetics of endothelin secretion: EDRF is very short-lived, whereas the effect of an intravenous bolus of endothelin lasts for an hour or more. EDRF is inactivated by binding to haemoglobin or to albumin and it is therefore likely that it acts only on the sub-adjacent vascular smooth muscle (and then only transiently), not on vascular smooth muscle to which it would be carried downstream by the blood. Whether there is any vectorial secretion of endothelin (to the luminal or abluminal surface of the endothelium) remains to be determined. Note that endothelium contributes to the control of vascular tone not only via

EDRF and endothelin, but also by secreting, metabolizing and responding to several classes of vasoactive agents — for example, arachidonate metabolites, peptides, amines, nucleotides and nucleosides (see ref. 12 for review). The metabolism of vasoactive agents can be either through uptake and intracellular transformation (for example, adenosine, some prostaglandins and some amines) or by the action of ectoenzymes (for example, nucleotides and some peptides), and the endothelial response to several vasoactive agents is the production of a different class of vasoactive agent — for example, the secretion of endothelin induced by adrenaline.

These examples are far from exhaustive, but they illustrate the range and diversity of vasoregulatory functions exhibited by the thin, fragile lining that is the endothelium. Despite the variety of

activities already demonstrated, it would be a brave person who would bet that the story of endothelial biology ends with endothelin. □

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## Oceanography

# Whither organic carbon?

W. G. Deuser

TWO-THIRDS of the particulate organic carbon (POC) carried by rivers ( $150 \times 10^{17}$  grams per year), most of it associated with clay minerals, is resistant to degradation in the terrestrial and fluvial environment, according to a report by Ittekkot on page 436 of this issue<sup>1</sup>. That amount is comparable to most current estimates of the rate of burial of organic matter in the entire ocean, including deltaic and shelf areas<sup>2</sup>. Does this suggest that all organic matter buried in the ocean is of terrestrial origin? If not, where does all the terrestrial carbon go? Does it reach the ocean and become oxidized there? If it is not oxidized, do marine sediments preserve mostly terrestrial carbon?

## Depositories

There is evidence that terrestrial organic matter is preserved in marine sediments, but the bulk of such material seems to be confined to the vicinity of continents. Estuaries and shelves are by far the most important depositories of organic matter in the sea<sup>2,3</sup> for two reasons: first, the supply there is the greatest, both from land and from marine production; and second, the high bulk sedimentation rate and oxygen demand in the water lead to rapid removal of the material from the oxidation zone at the sediment surface. As Ittekkot suggests<sup>1</sup>, however, the huge deep-sea fans extending from the Asian mainland could be as important to the burial of organic matter because the rivers forming them carry the greater part of all riverine POC. Part of their load of clay and associated resistant organic matter could be deposited far from the shore.

Present data do not place tight constraints on the burial rates in those fans.

An assumption pervading geochemical budget considerations of this kind is that snap-shot measurements of present-day conditions (be they river loads or concentrations in the ocean's water column) can be compared with quantities derived from measurements on surface sediments. Except for the topmost layers in (rare) anoxic environments, all surface sediments in the ocean are constantly 'ploughed' by bottom-dwelling animals and therefore represent a mixture of material deposited over periods as long as several millennia. Thus the best value for the present burial rate of organic matter in the ocean is, in reality, an average for the past few thousand years. If today's true rate was significantly different from that average, or if the nature of the material being buried during the past few thousand years was different from that of today's, any comparisons between present-day supply by rivers and long-term burial rates in the ocean could be inappropriate.

There are two striking differences between present conditions and average conditions over the past few millennia. First, continental deglaciation significantly changed vegetation patterns, the size of continental shelves, and sea level. Second, industry, agriculture, deforestation and river damming have altered river loads in qualitative and quantitative ways. For these and similar reasons, it has been argued<sup>4</sup> that there is an excess of supply and production of organic carbon in the coastal zone over near-shore consumption

and burial. But these arguments have largely been laid to rest<sup>5,6</sup>, at least to the extent that they do not prove that marine carbon storage has increased greatly during the past century.

## Discrepancies

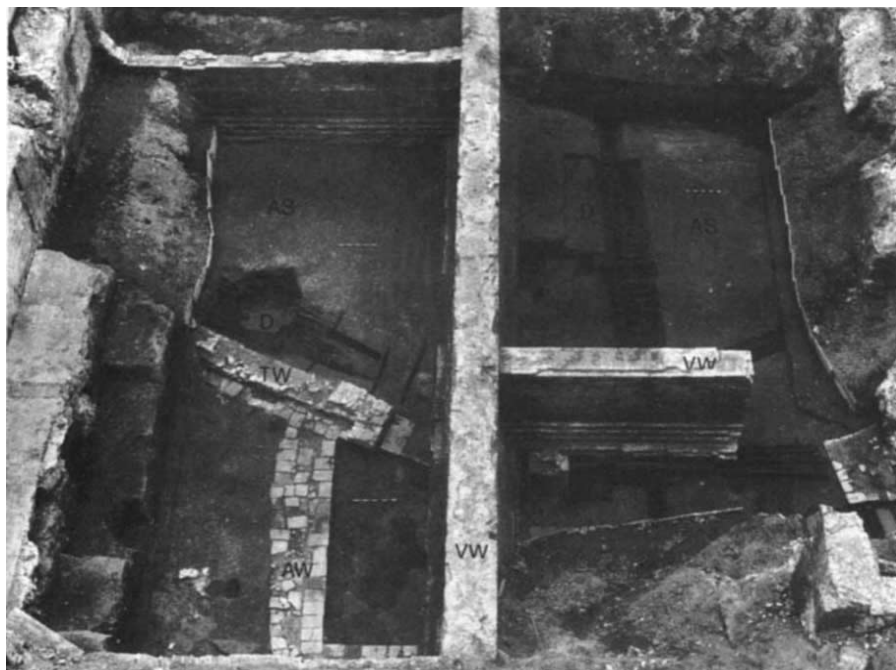
It seems to be easier to point out additional discrepancies in our understanding of the land-ocean carbon balance than to answer the questions raised by Ittekkot's report<sup>1</sup>. Besides their POC load, rivers carry an approximately equal load<sup>7</sup> of dissolved organic carbon (DOC) — about  $200 \times 10^{12}$  grams per year. If all this were to reach the ocean and if it were as resistant to degradation as is POC, it would take less than 3,000 years to 'fill up' the oceans' reservoir of DOC by rivers alone. But DOC in the deep Pacific is reported to have an average radiocarbon age of 6,000 years and is believed to be mostly (90 per cent) of marine origin<sup>8</sup>. Although many qualifications could be added, it seems that there is much more organic matter coming down the rivers than can be accounted for in the ocean. Where does it go?

The complex physical, chemical and biological processes at the interface between fresh and salt water in estuaries and deltas can only dispose of part of the load. Neither near-shore burial rates nor oxidation and attendant release of nutrients are high enough to accommodate the excess carbon. Deep-sea fans are one place to look for it. Another, more provocative, supposition is that the ocean is comparable to a heterotrophic organism. That is to say that it depends on an import of terrestrial organic matter to maintain its level of metabolism, because there is an excess of respiration over primary production of organic matter. Indeed, it has recently been suggested<sup>9</sup> that much of the near-shore ocean shows net heterotrophy. It could also play a role in the high productivity of river plumes. Waters originating in the Amazon River plume retain elevated concentrations of natural pigment for up to a year after their departure from the river mouth while drifting thousands of kilometres into the Atlantic<sup>10</sup>. The high production associated with these waters may in part be sustained by slow release of nutrients from land-derived dissolved organic matter. □

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# London's Roman amphitheatre



SEVERAL months of digging by archaeologists in the centre of London have just been rewarded by the discovery of the remains of the amphitheatre of Roman London. Close to the historic Guildhall, now the home of the Corporation of London, and at a depth of more than five metres, just a short section of the curved brick- and stone-built walls sufficed for the reconstruction of the plan of an elliptical arena about a hundred metres long. The photograph shows the main area of the excavation: VW, Victorian wall; D, drain; TW, inner wall of theatre; AW, wall of antechamber; AS, arena surface. The doorway in the wall of the theatre, leading from the antechamber to the arena, can be clearly seen.

The arena was not a towering mass raised on vaults, tunnels and arches like the Colosseum at Rome or the arenas of Arles and Nîmes. London's amphitheatre was enclosed merely by banks of earth on which temporary stands of seating were erected. Arenas of this type were known in many parts of the Roman empire, for example in Romania (Ulpia Traiana), Hungary (Aquincum), Austria (Carnuntum) and Germany (Xanten on the lower Rhine), and examples in Britain are found in Cirencester, and near the legionary fortresses at Caerleon in south Wales and Chester in north-west England.

The London arena lies next to the Cripplegate military fort, which may have accommodated army personnel seconded to the staff of the provincial governor, whose Thameside residence is now partly overlain by Cannon Street railway station. Conceivably, the amphitheatre began life as a special arena (*ludus*) that would have been used for military training and exercises, though in its main civil phase it was much larger than known examples of this type.

A city's amphitheatre was intended to house the great popular entertainments, gladiatorial combats, the hunting and killing of wild beasts and the execution of condemned persons. This last was held as generally having less entertainment value and was often reserved for the luncheon interval during the great festivals. Games were paid for by leading citizens, and although many of the educated classes affected to despise the vulgarity of the arena, it was their decision to sustain it that made the games such a distinguishing feature of Roman public life.

Londonium was a creation of the Romans, a flourishing port for merchants and commerce in the years following conquest by the emperor Claudius in AD 43. Yet little is known of its buildings and layout, apart from a great forum and basilica on Cornhill, beneath the line of Gracechurch Street, a temple of the Persian god Mithras, unearthed more than thirty years ago, and the encircling walls, two bathblocks and several mosaic pavements which were discovered in the nineteenth century. It is to be hoped that some of the arena will be preserved for permanent exhibition, a reminder not only of how much (or possibly how little) tastes have changed, but also of a Roman origin which London shares with many of the principal cities of Europe. (Photograph courtesy of Jan Scrivener/Museum of London.)

John Wilkes

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## Vertebrate palaeontology

## New pterosaurs from Brazil

David M. Unwin

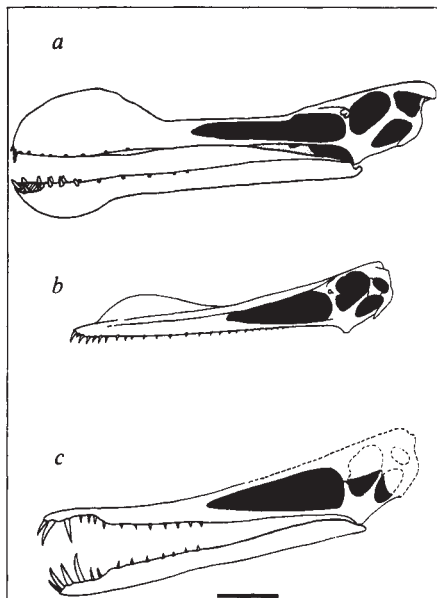
PTEROSAURS took to the air in the late Triassic about 225 million years ago, preceding birds and bats by over 70 million years. The pterosaur skeleton, like those of birds and bats, was light and delicate, as required by a flying vertebrate, but quite unsuitable for fossilization. The fossil record of pterosaurs is consequently very patchy. Upper Triassic and Jurassic rocks have yielded a number of well-preserved

and echinoids) together with vertebrates (fish, turtles and crocodiles) and plants have already been identified from what is thought to have been a large, virtually landlocked lagoon<sup>7</sup>. In the early 1970s part of an articulated pterosaur wing was found in a concretion from a fossil-fish horizon. Many more pterosaur specimens have been collected and described subsequently. The bony remains are extremely well-preserved, uncrushed and often free of distortion. In one exceptional case<sup>6</sup>, part of a wing membrane has been preserved.

The pterosaurs, probably all fish eaters, varied in size from between 2 and 6 metres in wingspan, with skulls up to 0.6 metres in length. Ten species have been described so far, but may not all be valid. Although some species are represented by skulls, others such as *Araripedactylus dehmii*<sup>8</sup> and *Santanadactylus brasiliensis*<sup>9</sup> are based on

isolated postcranial remains and may be synonymous with those based on skull material. Despite the likely exaggeration in numbers of species, it is clear that pterosaur diversity was high during the Aptian stage. At least four pterosaur genera, *Tropeognathus*<sup>5</sup>, *Anhanguera*<sup>3</sup>, *Cearadactylus*<sup>2</sup> (a–c, respectively, in the figure) and *Araripesaurus*<sup>1,4</sup> can be distinguished. *Araripesaurus* differs from a fifth genus *Santanadactylus*<sup>4</sup> only in possessing a vertical nasal process (possibly missing in the skull of *Santanadactylus*) and in a lack of fusion between its bony elements (possibly a pathological or pedomorphic feature), so that these forms may be congeneric.

*Araripesaurus* resembles the poorly known pterosaur *Ornithocheirus*<sup>10</sup> from the late Lower Cretaceous Cambridge Greensand of England. Inclusion of *Araripesaurus* within the family Ornithocheiridae<sup>1,4</sup>, if correct, would support a close relationship between ornithocheirids and pteranodontids. *Araripesaurus* and *Pteranodon* share a number of advanced characters such as the virtual exclusion of the squamosal bone from the post-temporal opening of the skull.



Three pterosaur skulls from the Santana Formation of north-eastern Brazil illustrating the great diversity of Lower Cretaceous pterosaurs. a, *Tropeognathus* as preserved; b, reconstruction of *Anhanguera*; c, reconstruction of *Cearadactylus*. Scale bar, 10 cm. (Redrawn from ref. 6.)

pterosaurs including *Dimorphodon*, *Rhamphorhynchus* and *Pterodactylus*. But the Cretaceous period has been less productive, yielding just a handful of often poorly preserved species, such as *Dsungaripterus*, *Pteranodon* and *Quetzalcoatlus*. A new pterosaur fauna<sup>1–5</sup> from the Lower Cretaceous Santana Formation of Araripe in north-eastern Brazil is thus of particular interest. The Santana pterosaurs represent a vital addition to the Lower Cretaceous fauna and establish Araripe as one of the most important pterosaur localities ever discovered.

The Santana Formation, a thick sequence of limestones and shales outcropping on the Araripe Plateau in north-eastern Brazil, contains a rich and well-preserved fossil fauna. Many invertebrates (bivalves, ostracods, crustaceans, insects

## Charles Glen King (1896–1988)

CHARLES Glen King, who died on 23 January, was one of the world's most eminent investigators in the field of nutrition. He was a man of exceptional character and ability. He was modest, humble, religious and a hard worker. He had, like Charles Darwin who wrote concerning his own success in science: "a love of science, unbounded patience in long reflecting over any subject, industry in observing and collecting facts, and a fair share of invention as well as common sense."

King was best known for his researches from 1928 to 1932 on the isolation and identification of vitamin C. In my opinion, both King and Szent-Gyorgyi deserve equal credit for their almost simultaneous discovery of vitamin C, and the Nobel prize should have been awarded jointly, not just to Szent-Gyorgyi (see the letters from Thomas H. Jukes and Ronald Pethig on page 390 of this issue). Both King and Szent-Gyorgyi worked resourcefully and independently, at least during 1932.

King was professor of chemistry at Pittsburgh from 1930 to 1942. He then left Pittsburgh to become scientific director and then president of the Nutrition Foundation in New York City. He retired from the foundation in 1963 and became associate director of the Institute of Nutritional Sciences at Columbia University, serving as a part-time consultant to the Rockefeller Foundation and to various United Nations agencies.

In his career with the Nutrition Foundation, King supported nutrition research and the development of nutrition policy,

largely through the Food and Nutrition Board of the National Research Council of which he was a member from its beginning in 1942 to 1970. King was instrumental in bringing together, in harmony and common purpose, leading individuals in food and nutrition from industry, government and academia.

King, at the Lind Bicentennial Symposium at the University of Edinburgh in May 1953, said "... one point is intriguing to a student of nature; did the loss of one or more genes that permit biological synthesis of the antiscorbutic vitamin, without disrupting other structures essential to survival, occur only once, or more often in nature? Assuming a common heritage of man and other surviving primates, can it be that we, among all the higher forms of animals and plants, share the single successful experiment with guinea-pigs, in the process of evolution?" He continued: "In place of death and the scourge of a disease that had plagued mankind since long before the dawn of history, he [Lind] gave to his generation and ours the delights of orange juice for breakfast, lemonade on hot summer days, fresh salads at dinner, and a new, higher concept of normal health. He freed men's bodies to travel the high seas at full vigour. But perhaps of greater importance, he set their minds to travel more freely in the realm of truth."

Fredrick J. Stare

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The skull of *Tropeognathus*<sup>5</sup> presents a bizarre appearance. The cranial region is similar to that of other Santana pterosaurs but the anterior end of each jaw bears a large, deep and narrow keel (*a* in the figure). The keels may have acted as stabilizers during feeding<sup>5</sup> wherein the tips of the jaws were trawled through the water surface, in a similar fashion to the modern skimmer *Rhynchops*. It is unclear why *Tropeognathus* needed stabilizing keels, as other fish-eating pterosaurs appear to have managed without them. *Tropeognathus* bears quite a striking resemblance to the poorly known *Criorhynchus*<sup>10</sup> from the Cambridge Greensand, the fragmentary remains of which have until now been very puzzling.

The greatly enlarged front teeth of *Cearadactylus*<sup>2</sup> (*c* in the figure) are reminiscent of the fish-grabbing jaws of *Dorygnathus*, a Lower Jurassic rhamphorhynchoid from the Cambridge Greensand, further highlighting the many similarities between the Cambridge Greensand and Santana forms.

The Santana form offers much hope for future studies of the evolutionary history of Cretaceous pterosaurs, hampered so far by lack of material. A recent study based on neck vertebrae<sup>11</sup> indicates the existence of two separate lineages of Cretaceous pterosaur: one with short necks culminating with the crested pterosaur *Pteranodon*; and one with long necks culminating with the giant *Quetzalcoatlus*.

But further resolution of the evolutionary history of Cretaceous pterosaurs will require more characters than provided solely by neck vertebrae. Future studies are likely to focus heavily on skulls because they provide plenty of taxonomic characters and avoid the problem of relating taxa defined using postcranial remains to taxa defined on the basis of skull material. Whatever the basis of these studies, the superb preservation and relative abundance of the Santana pterosaurs guarantees them a prominent position in pterosaur biology. □

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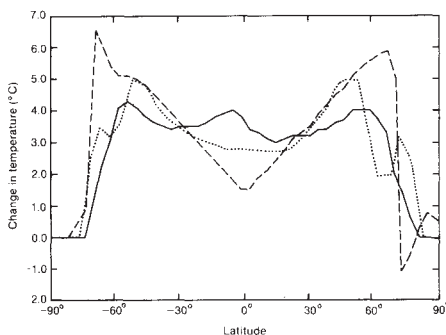
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## Climatology

# Local effects of greenhouse gases

J.F.B. Mitchell

THE increase in the atmospheric concentration of radiatively active (greenhouse) gases since 1860 may have raised the global mean surface temperature by 0.5 °C or more<sup>1,2</sup>, and the projected concentrations of these gases could produce a further warming of 1.5 °C over the next 40 years<sup>3</sup>. The exact magnitude of the warming is uncertain, mainly because of uncertainties in the strength of the feedbacks involving changes in cloud cover and cloud radiative properties<sup>4</sup> and the extent to which the thermal inertia of the oceans slows the rate of the expected



Changes in sea-surface temperature, June to August, based on CO<sub>2</sub> doubling experiments. Solid curve, Rind's experiment 'CON'; dashed curve, Rind's experiment 'ALT' based on GFDL work<sup>4,9</sup>; dotted curve, our experiment. The curves are normalized to give a global mean temperature change of 4.2 °C.

warming<sup>1,2</sup>. In addition, both the low horizontal resolution of climate models and discrepancies in the regional changes in climate that they simulate<sup>4,5</sup> preclude a detailed assessment of the socio-economic impact of the changes in climate. In a recent paper<sup>6</sup>, Rind attempts to identify some of the reasons for the discrepancies in the regional climate change simulated by different models. Although he shows that amplified changes in sea surface temperatures at high latitudes may be important in this respect, there is still much to be explained.

The radiative effects of increasing the concentrations of the different greenhouse gases are qualitatively similar, giving a warming of the troposphere and surface and a cooling of the stratosphere<sup>3</sup>. Thus the climatic effects have been assessed using CO<sub>2</sub> as a surrogate for the other gases. The potential effects can be estimated by running three-dimensional climate models to equilibrium with present-day and doubled CO<sub>2</sub> amounts, and comparing the time-averaged statistics of the two simulations. The atmospheric part of the model can be regarded

as a numerical weather-prediction model modified to include those processes that are important on a climatic timescale. The ocean is represented by a well-mixed layer of prescribed depth which may or may not include the effect of transport of heat by ocean currents.

One way in which calculations differ is the extent to which the surface warming is enhanced in high latitudes. Simulations carried out at the Geophysical Fluids Dynamics Laboratory (GFDL) at Princeton<sup>4,7</sup> indicate that increased CO<sub>2</sub> substantially enhances warming in high latitudes and consequently reduces the meridional (north-south) gradients of sea surface temperature. On the other hand, simulations carried out at the Goddard Institute for Space Studies (GISS) in New York<sup>8</sup> predict little change in meridional gradient. To isolate the influence of the changes in sea temperature, Rind investigated the effect of prescribing temperature changes similar to those found in the GFDL and GISS experiments using the same high-resolution version of the GISS model and the same global mean warming in each case (see figure).

In Rind's control simulations using prescribed present-day CO<sub>2</sub> concentrations, sea temperatures and the extent of sea ice were prescribed from climatological values, whereas in the two perturbed experiments, CO<sub>2</sub> concentrations were doubled and sea-ice extents were altered to be consistent with the prescribed change in sea temperatures. In the experiment with little change in meridional gradient and hence greater equatorial warming ('CON', based on the GISS work<sup>8</sup>; see figure) the intensities of several features including low-latitude precipitation, the Hadley cell (a vertical circulation cell at low latitudes) and the sub-tropical jet stream increase with respect to the control run. In contrast, the intensities of these features decrease in the experiment with the greater warming in high latitudes ('ALT', based on the GFDL work<sup>4,7</sup>; see figure). Note that the changes in low-level air temperature increase with latitude in both experiments, though more so in ALT.

Rind's work demonstrates that the sea-surface temperature changes are crucial in determining other aspects of the simulated response, but does not attempt to address the problem of why the temperature changes are different. Oceanic heat transport was ignored in the GFDL study but was prescribed in the original GISS experiments<sup>8</sup>. Our recent study<sup>5</sup> at the UK Meteorological Office that used a



parametrization of the transport of heat by ocean currents similar to that used by GISS, however, also finds a pronounced decrease in the meridional sea temperature gradient on doubling the amounts of  $\text{CO}_2$  (see figure). Hence the reason for the discrepancy seems to lie in the atmospheric models. The GFDL and GISS models differ in many ways so it will be difficult to identify why they produce such a different response.

Rind also finds differences in the regional response in the two experiments. For example, the melting of ice over the Greenland and Antarctic ice sheets and the drying of the mid-latitude continents in summer are more pronounced in the ALT than in the CON calculations. The accurate determination of these changes is crucial for the evaluation of the effect of greenhouse gases on sea level and agriculture, respectively. A pronounced summer drying of the mid-latitude continents was also reported in the original GFDL experiments<sup>7</sup> and in our experiment<sup>5</sup>. Other features are known to influence the drying of the land surface in summer, however, and recent work<sup>9,10</sup> shows that the degree to which the soil becomes saturated in early spring and the treatment of snow melt are also important.

Rind notes<sup>6</sup> that improving the horizontal resolution of models gives more realistic results for many (but not all) features of the present-day climate. The original GISS experiments<sup>8</sup> were run using an  $8^\circ \times 10^\circ$  latitude-longitude grid which does not resolve a large proportion of the mid-latitude depressions that are an important feature of the real climate. Furthermore, the area of a grid square in mid-latitudes is greater than that of France, limiting output detail. The changes in precipitation in Rind's experiment, using a  $4^\circ \times 5^\circ$  grid, show some marked differences from those obtained at lower horizontal resolution. For example, summer precipitation is reduced over the central United States and around the Mediterranean in the CON experiment, relative to the control but not in the original low-resolution GISS study. (The CON simulation, however, was not quite in thermal equilibrium as the sea-temperature changes were prescribed.)

All studies of the detailed climatic effects of increasing  $\text{CO}_2$  have so far been based on atmospheric general-circulation models coupled to a mixed-layer ocean and run to thermal equilibrium. Thus, the influence of the deeper ocean and changes in ocean circulation have been ignored. The large thermal inertia of the ocean will slow the rate of warming caused by the increase in greenhouse gases. The observed global mean surface temperature has warmed by about  $0.5^\circ\text{C}$  since 1900, but was also probably  $0.2\text{--}0.3^\circ\text{C}$  warmer during the previous 50 years<sup>11-14</sup>. Thus, the actual warming to date is, as

expected, much smaller than the estimated equilibrium warming of  $0.7\text{--}2.3^\circ\text{C}$  (refs 3,4) but falls within the lower end of the range of estimates that allow for oceanic thermal inertia<sup>1,2</sup>. The apparent agreement between the overall observed and predicted rises in global temperature since 1900 may be fortuitous, however, for the pattern of changes since 1900 cannot be ascribed to greenhouse gases alone.

It is possible that because the thermal inertia of the oceans varies with latitude, the change in meridional temperature gradient during warming will differ from that achieved once the warming has reached equilibrium; as Rind shows, this will lead to differences in the regional distribution of climate change. Changes in ocean circulation can also produce large changes in local climate, as during the last ice age when the flow of warm surface waters from the Gulf Stream into the north-east Atlantic was diverted southwards<sup>15</sup>. This change was apparently accompanied and perhaps even reinforced by changes in the deep-ocean circulation. The modelling of the global oceans is still in its infancy, however, and details of regional climate change simulated using coupled ocean-atmosphere models have yet to be published.

There are many uncertainties in the modelling of climate change which remain to be narrowed before the economic impact of increases in greenhouse gas concentrations can be assessed reliably. The physical and biological processes involved in climate change are not yet modelled adequately. A prime goal of current research is to include realistically the effects of the ocean as well as upgrading the atmospheric and land-surface component of climate models. In the meantime, although the simulated changes in regional climate from current climate models are of considerable interest, they cannot yet be relied on for making quantitative estimates of economic, agricultural or social impacts. □

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## Daedalus

# Stopping the rot

COPPER, nickel, chromium and many other heavy metals are, in high concentrations, toxic to both animals and plants. Indeed, one standard method of preserving wood against insect and fungal attack is to treat it with compounds of copper. And yet some plants have evolved to tolerate, and perhaps even need, heavy metals. Shrubs like *Becium homblei* and woody species like the *Combretaceae* manage to grow in the copper-rich soils of the mining districts of Africa, and *Hybanthus floribundus* in Australia flourishes in nickel-rich soil where almost nothing else will grow. These plants can accumulate several per cent of heavy metal in their tissues, apparently by chelating them into stable compounds which do not interfere with the plant's metabolism.

Daedalus sees great possibilities in this biochemical trick. DREADCO's biotechnologists are studying the genetic constitution of metal-accumulating plants, and crossing them with other woody species. The idea is to establish metal-accumulating ability in big conifers.

The direct route to this goal is, of course, genetic engineering. Bacterial genes have recently been transferred to tobacco plants, and metal-binding genes have been transferred from hamsters to oilseed crop plants, so metal-chelating chemistry might well be transferred from one plant to another. But even if this turns out to be unfeasible, a conventional selective-breeding programme should work. In areas afflicted by industrial pollution, some local plant communities have evolved metal-tolerance in only a few decades; and fast-growing conifers that can grow to maturity in a few years are already being developed. So a copper-rich fir tree could be developed quite quickly.

The new tree will have two great advantages. First, it will grow in metal-polluted industrial wasteland, usefully reclaiming it while freeing more valuable land elsewhere. Second, it will yield naturally rot- and insect-proof timber. Decay, the curse of all timber construction, will be beaten at source. Wood, that light, cheap, strong, easily worked material, will at last be trust worthy even in humid, rainy and termite-infested regions of the world.

Heavy-metal trees should be easy to grow. They may need 'fertilizing' with metallic industrial waste; but that should discourage rival plants, while insect pests and fungal diseases will be no problem anyway. Rot-proof and everlasting, they will command high prices on the international timber market. And when at last a metallized-wood structure is broken up and burnt, its charcoal may well smelt its metallic content directly to nuggets of pure metal in the ashes.

David Jones

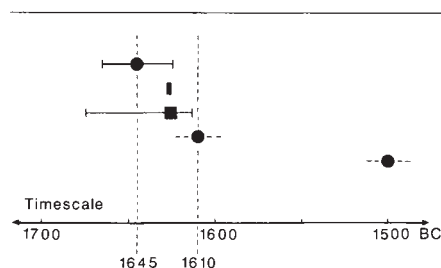
## Dating of the Santorini eruption

SIR — I would like to comment on the radiocarbon dating section of the recent paper by Hammer *et al.*<sup>1</sup>, and particularly on the comments in the accompanying News and Views article by Cadogan<sup>2</sup>, on dating the Santorini eruption.

First, the conclusion of Cadogan, that "Taking everything into account, a date of the sixteenth century BC seems the most likely", is not correct. Only traditional pottery studies support this view<sup>2-4</sup>. Moreover, there are several plausible contrary interpretations using traditional archaeological evidence (refs 5, 6). All other evidence currently points to a date of the later seventeenth century BC<sup>1,5</sup>. Cadogan argues that because three independent scientific techniques (ice-core, dendrochronology and radiocarbon) produce results which vary by only 30 years over 3,600 years, they should all be rejected. Instead, he asks us to accept a subjective archaeological date a century later: a date derived from the disputed comparison of art designs, or correlations from dubiously datable finds, with Egyptian pharaonic dates (themselves debated).

Concerning the radiocarbon evidence, where it is not possible to raise doubts of relevance, further analysis is desirable. Hammer *et al.*<sup>1</sup> consider a set of 14 short-life dates associated with the destruction of the site of Akrotiri on Thera (Santorini). These dates should provide a range shortly before the eruption, which engulfed the site. They reject only three dates as outliers, and thus include for analysis three dates from undersized samples with their attendant large errors, and some others which even a glance at their Table 1 suggests are aberrant (P-2792, P-2794). The unstated result is that their weighted average of  $1,359 \pm 17$  carbon-14 yr BC has an acceptable, but uncomfortably high test statistic ( $T'$ , following ref. 7; with  $\pm 14$  yr calibration error — allowed for here and not again at calibration), of 15.12, compared with  $\chi^2_{13,0.05} = 22.36$ . If a statistical clustering procedure were used<sup>8</sup>, then of the 17 original dates, a central set of 12 dates (excluding P-2792 and P-2794 from Table 1 of ref. 1) is derived, with a weighted average of  $3,321 \pm 18$  yr before present (BP) ( $T' = 4.1 < \chi^2_{11,0.05} = 19.7$ ). The calibrated date<sup>9</sup> is about 1622 BC, the range  $\pm 1\sigma = \sim 1639-09, 1552-45$  BC. Moreover, if the two remaining dates from undersized samples were removed (P-2793, P-2795), together with the least coherent date of the above twelve, P-1885 (which is the only date not to include the weighted average within its  $1\sigma$  error) then a significant, coherent group of nine (of seventeen) dates emerges with a weighted average of  $3,331 \pm 20$  yr BP. The calibrated age is about 1629 BC, the range at  $\pm 1\sigma =$

$\sim 1675-12$  BC. The weighted averages of Hammer *et al.*<sup>1</sup> (and of Aitken<sup>10</sup>) are thus too low because they uncritically include the aberrant ( $> 2.5\sigma$  deviation) low, small standard error (and hence statistically biasing) date of P-2794 (also P-1885; a problem only partly countered by Hammer *et al.* by the inclusion of the aberrantly



Graphic representation of the different chronological evidence relating to the calendar BC date of the Bronze Age eruption of the Santorini volcano. From the top, symbols indicate: ice-core date<sup>1</sup>; frost-ring date<sup>12</sup>; radiocarbon; revisionist archaeological<sup>5</sup>; and traditional archaeological<sup>3,4</sup>.

high P-2792, because of its statistically mitigating large  $\sigma$ ).

The calibrated date ranges derived above clearly indicate that the eruption occurred in the seventeenth century BC (see also ref. 11). Moreover, in the former case, the small mid-sixteenth century BC date range is statistically unlikely, as the appropriate section of the calibration curve mainly lies outside the total  $\sigma$  boundaries of the weighted average<sup>9</sup>.

Hence, regarding the dating of the Bronze Age eruption of Santorini, I conclude as follows. Frost-ring damage suggests a date of 1628–26 BC<sup>12</sup>. Radiocarbon date analysis supports an entirely compatible seventeenth century BC date range of about 1675–09 BC, with the calibrated mean at about 1629–22 BC (see also ref. 11). Ice-core analysis suggests a date range of  $1645 \pm 20$  BC<sup>1</sup>, again consistent within its very small (over 3,600 years) error margin. Recent re-interpretations of the archaeological evidence<sup>3,6</sup> suggest similar date ranges. Only the traditional archaeological interpretation is at odds (see refs 2–4). Taking everything into account, the most likely date of the eruption is the later seventeenth century BC. Thorough re-consideration of the (inconsistent) traditional Aegean Bronze Age pottery chronology now seems necessary. It is to be hoped that dates from atomic mass spectrometry soon to be obtained from seeds on Thera, will finally resolve this controversy.

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HAMMER *ET AL.* REPLY — We do not completely agree with Manning's comments on our treatment of the radiocarbon dates<sup>1</sup>. Certainly one can treat the data further statistically, and also try to exclude further dates, but only at the risk of introducing a bias in the selection. We took the conservative view of excluding only the three obvious outliers. It is thus not justified to exclude dates made on undersized samples just because they are undersized and have a large error. If weighted means are calculated, such dates only carry little weight ( $P = 1/\sigma^2$ ), but they ought to be included. This applies for instance to P-2792 "which even a glance at their Table 1 suggests are aberrant". The date is not aberrant by normal statistical evaluation; it deviates by  $2\sigma$  from the calculated mean, which is an expected deviation in a set of 14 dates.

If the exclusion of radiocarbon dates is carried as far as proposed by Manning, and also by Betancourt and Michael<sup>11</sup>, we arrive at a set of nine dates, which no longer show a normal statistical scatter, as all the remaining dates are within  $1\sigma$  from the mean. It was to avoid such a situation, which might be criticized as biased, that we restricted our statistical treatment of the radiocarbon dates, although we were aware that additional exclusion of dates would give a mean radiocarbon age in closer agreement with our ice-core date.

The main purpose of our paper was to show that ice-core dating, radiocarbon dates, and a tree-ring (frost-ring) date all point to a seventeenth century BC date for the Santorini eruption. Of these we consider the ice-core date as the most probable, because the acidity signal in the 1645–44 BC ice layers is clearly related to a major volcanic eruption, whereas the frost damage at 1628–26 BC, though carrying a statistical probability of being related to a volcanic eruption<sup>12</sup>, could have been caused by climatic impacts other than volcanism. We consider it premature to fix the date more precisely than in our paper, by radiocarbon or other methods. This is why we left a question mark at the proposed date of  $1645 \pm 7$  yr BC.

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CADOGAN REPLIES — My remarks in my News and Views article<sup>2</sup> on the paper by Hammer *et al.*<sup>1</sup> were too cryptic. I will go through the arguments again. First,



Thera's (Santorini's) is the sole Plinian eruption known at present of the mid-second millennium BC. Second, other such eruptions at that time elsewhere in the world cannot be excluded. Third, there is no certainty then that the 1645 BC acid fallout in the Greenland ice core or the 1628–26 BC frost damage in Californian bristlecone pines relate to Thera, and to no other eruption.

Fourth, so only the third independent scientific technique (radiocarbon) is safe for Thera. I followed the average range calibrations of Hammer *et al.*<sup>1</sup> and Aitken<sup>10</sup>, which are compatible both with a seventeenth-century BC date for the eruption and with a (slightly raised) usual date in the sixteenth century. Although, as Manning shows, statistics suggest that the seventeenth century is more probable, the weight of archaeological evidence, reviewed most recently by Warren<sup>4</sup>, still supports the sixteenth century date.

Finally, Warren raises the start of the Late Minoan IA/Late Cycladic I phase slightly, to 1600 BC or a little earlier. The eruption happened at, or towards, the end of this phase: the usual (I prefer the neutral word to Manning's word "traditional") date has been around 1500 BC. To revise this terminal date slightly upwards is compatible with the strong archaeological evidence for the dating of the Aegean Late Bronze Age<sup>4</sup>, and produces a date in the second half of the sixteenth century (as I argued<sup>2</sup>) that is equally compatible with calibrated radiocarbon dates as seen by Hammer *et al.*<sup>1</sup> and by Aitken<sup>10</sup>.

I conclude that it cannot be a case of archaeologists contradicting three independent scientific methods, when it is not yet certain that two of the methods apply while the third still allows a (somewhat raised) usual archaeological date.

If, however, it is possible sometime to exclude other potential sources for the Greenland/California phenomena, then everyone must accept a seventeenth century date and, as Manning suggests, re-examine yet again Aegean pottery chronology. That is, of course, something that is happening constantly, often under the stimulus of new discoveries, archaeological and scientific. It is in the honourable tradition of Aegean archaeology to revise.

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## Evolution of the third kind

SIR—I did not expect when I described the evidence and made some proposals<sup>1</sup> concerning the contribution of mechanisms of DNA turnover (such as unequal crossing-over, gene conversion, slippage and transposition) to the discrepancy between interspecific divergence and intraspecific polymorphisms of the *Adh* gene in *Drosophila*<sup>2</sup>, to read a reply<sup>3</sup> that turns observation into speculation and redefines drift and selection so as to accommodate all new processes into this happy duo.

DNA slippage is operating in the 5'-flanking region of the *Adh* gene because the entire region is rich in A+T, has frequent runs of homonucleotides and many small insertion/deletions<sup>4</sup>. No matter how many insertion/deletions are artificially eliminated by sequence alignment procedures, it is not possible to dismiss the major effects of slippage on sequence diversity in this region.

Greenhough and Harvey<sup>5</sup> twist this factual statement into a Talmudic polemic to the effect that if slippage were not operating then I would assume that the 5' regions diverge less than silent sites of exons, which supposes selection is operating, and given that no selectively

constrained functional sequences exist in 5' regions then I am wrong. I made no such assumption. Equivalent levels of interspecific divergence in 5' regions and exons could mean that either slippage operates in *Adh* exons too, as in many other genes<sup>5</sup>, or that codon usage and sequence context are constraining silent site changes.

Slippage, coupled to the possibility of a domain of unbiased gene conversion covering the 5' region, could provide an answer why the 5' regions are fourfold less polymorphic than the gene. By contrast, selection may be promoting higher levels of gene polymorphism, in particular in the third exon<sup>6</sup>. Greenhough and Harvey argue against the slippage plus conversion hypothesis on the grounds that conversion would need to influence "both flanking regions, both introns and two exons, but not the third exon!". It is frequently observed, however, that the unit lengths, rates, biases and polarities of conversion can differ both within and around genes (for examples, see refs 7–9), leading to different levels of polymorphism/divergence along the length of a gene.

There are abundant formal models that recognize the additional molecular means

for spreading variant sequences in populations. The widespread observation of concerted evolution (high intraspecific homogeneity despite interspecific divergence) also supports this mode of evolution. Such models and observations (see refs 9, 10, and references therein) contradict Greenhough and Harvey's expectation (unsupported by any formal model of their own) that alleles undergoing diffusion due to both genomic turnover and genetic drift would show increased rates of divergence.

Astonishingly, Greenhough and Harvey write that unbiased and biased conversion *are* drift and selection, respectively, at the molecular level, and that no further formal considerations are required. The spread of variant genes through a population by internal genomic forces is operationally distinct from that effected by the external ecological forces that underlie genetic drift and selection, and thus requires additional considerations for a full model of evolutionary dynamics.

I have always been perplexed over the need for Maynard Smith's fatherly reminder in his summary of our book<sup>11</sup>, cited by Greenhough and Harvey, "that people have been thinking about evolution for a long time". The turnover mechanisms that underpin molecular drive have been defined from the beginning<sup>11,12</sup> as an additional third, not a sole alternative, means for long-term population changes.

I agree with Greenhough and Harvey that "there is no mysterious third force". The evidence for it is alive and well, and even the innocent *Adh* gene in an obscure insect cannot escape it.

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## Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of less than 500 words and five references. □

# Echoes of the ancestors

Mark Ridley

**The Evolution of Individuality.** By Leo W. Buss. Princeton University Press: 1988. Pp.201. Hbk \$40, £25.10; pbk \$12.95, £8.10.

IN *The Evolution of Individuality*, Leo Buss suggests an original, evolutionary, explanation of development. He considers the kind of selective forces that would have operated during the early stages of the evolution of multicellular organisms, and interprets the course of development in terms of them. This leads him to concentrate on selection between lineages of cells, within the organism. In modern 'higher' organisms, this kind of selection is unimportant. The life cycles of these species are Weismannist, in the sense that they have a separate cell line for reproduction (the germ line), distinct from the rest of the body. This separate germ line enforces, over evolutionary time, a community of interest on all the cell lines of the organism: even if one line mutates, becomes cancerous and out-reproduces the other cell lines of the organism, the variant will usually die with the organism it will have helped to kill off.

However, as Buss begins by demonstrating, not all species have Weismannist life cycles. Indeed only a minority do; and the early ancestors of multicellular organisms presumably did not. In those ancestral forms, selection among cell lines would have been taking place. Buss seeks to show how the course of development in modern Weismannist species can be understood as the vestige of ancestral selective competition among cell lines. "The thesis developed here [he says] is that the complex interdependent processes which we refer to as development are reflections of ancient interactions between cell lineages in their quest for increased replication." It is a fascinating new approach to the problem of evolutionary embryology.

His most successful interpretation is of gastrulation. During gastrulation, cells multiply inwards from the surface of the embryo. Why should this happen? In crude summary, Buss's suggestion is this. The ancestral (like many modern) gastrulas would have swum in the sea, powered by cilia or flagella. Now, as Lynn Margulis has pointed out, it seems to be impossible for a cell in a multicellular organism both to produce cilia and to divide. The reason is not really known, but the consequence is that a cell lineage that stopped producing cilia should be able to divide faster. In the ancestral gastrula, such a lineage would have disrupted movement if it proliferated on the surface of the embryo. It would therefore have had to gastrulate

into the centre of the sphere.

Buss makes many other suggestions of the same general kind. Epigenesis, induction and inhibition, for example, are just the kinds of processes that could evolve in a system of competing cell lineages. Some of the suggestions, not surprisingly, are more plausible than others. I think his explanation of gastrulation may well be right; but I am more dubious about his theory of maternal control.

He interprets the maternal control of development as a method of reducing organismal disruption by selfish cell lineages. If the embryo itself controlled development, it would be "potentially susceptible to behaviour on the part of its constituent cell lineages which favor their own replication at the expense of the viability of the individual". That, however, is only true if the maternal control of metabolism also prevents the selfish mutation. I do not see why mutations could not arise that over-rode maternal control. Then Buss's argument would not work.

The book offers interesting ideas too on such related topics as sex, ploidy and units of selection. Here again, the ideas are always stimulating, but not always

convincing. For instance, Buss says he is not surprised by "the limited extent of secondarily derived asexuality", because "an organism with germ line sequestration can reproduce asexually only by modification of one stage in ontogeny — in that brief window between fertilization and the terminal determination of the germ line". But if that were a real difficulty, *all* evolution, not just the evolutionary loss of sex, would be impossible. All mutations have to arise in the germ line, and most have to exert their effects during an appropriate short interval in development.

Buss does tend to ignore, or caricature, positions that differ (or he says differ) from his own. He is especially concerned to advance a revolution in something called the "Modern Synthesis". That synthesis, we are told, was concerned only with selection at the individual level and even "explicitly excluded" suborganismal selection. If the Modern Synthesis was as daft as that, Buss is not the only evolutionary biologist who should be preening his revolutionary feathers. Moreover, there is a large literature on the topics Buss has discussed, most of which he does not even mention. He knows the literature all right, and at some points, when he is clearly writing against it, only experts will appreciate what he is saying; but generally this is not a problem. The book contains so many stimulating ideas on evolution and development that I believe almost any biologist would enjoy reading it. □

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## Enlightenment in polymer science

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**Mechanisms of Photophysical Processes and Photochemical Reactions in Polymers: Theory and Applications.** By Jan F. Rabek. Wiley:1987. Pp.756. £100, \$204.

POLYMER photochemistry continues to be a rapidly expanding field, and this book provides a comprehensive and in-depth account of the subject. With over 4,000 listed references, it is a remarkable compilation, reflecting the tremendous amount of work carried out in this area over the past 25 years. The reactions that occur in polymer media as a result of absorbed light are gathered into systematic groups and are all thoroughly discussed from a mechanistic point of view. A number of the principal applications of these reactions are also considered in connection with present and future industrial developments.

The book is divided into 16 chapters. After introducing the reader to the fundamentals of light-induced transitions in organic molecules and electronically excited states, Rabek discusses some basic photophysical processes, such as energy transfer and migration in macromolecules and the formation of excimers and exciplexes in aromatic polymers exposed to ultraviolet light. He concludes this section by describing some applications of fluorescence and phosphorescence spectroscopy to polymer science.

The next chapter deals in some detail with the most important topic of photo-initiated polymerizations, which can proceed via radicals, cations or charge-transfer complexes formed by photolysis of efficient initiators. Emphasis is given to the various reactions that are induced by light in the manifold photoinitiators which have been developed over the past few years, and altogether this chapter constitutes a welcome review of our present understanding of the elementary processes occurring in such photosensitive molecules. The surface modification of polymer materials through photografting,



and the synthesis of polymers through photocycloaddition reactions, are two other aspects of the subject that are briefly covered in separate chapters.

Although they have been comparatively neglected, photochromic polymers remain of great interest because of their potential applications. The mechanisms of the photoisomerization of polymers containing different types of photochromic groups are thoroughly discussed, in connection with the distinct properties of such materials.

Photocrosslinking and photocuring of polymers are among the fastest-growing areas in polymer photochemistry because of the widespread applications of these technologies, in microlithography for the formation of relief images, and in the surface treatment of materials by instant curable protective coatings. Rabek gives a comprehensive account of the basic chemistry involved in the different types of photoresist materials currently employed, but he covers only rather briefly the photocuring process which has recently come to prominence.

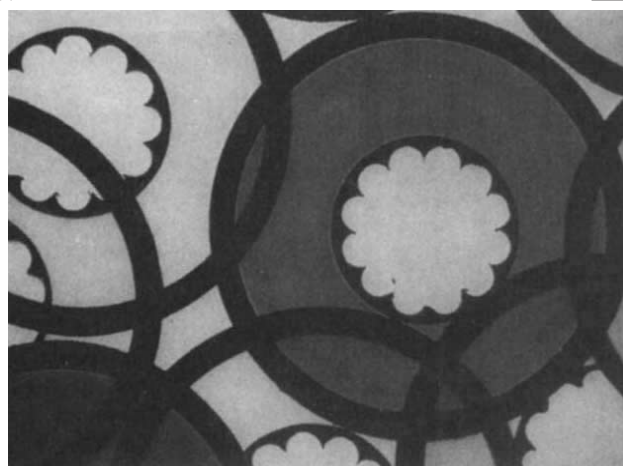
The last section of the book deals with the photodegradation of commercial polymers, and considers the methods used to improve the outdoor durability of these materials through the addition of light-stabilizers and antioxidants. These two chapters are mainly orientated towards basic mechanisms and product identification, and they describe the various chemical effects of ultraviolet radiation on polymers and the modes of action of a great number of photostabilizers. This is a helpful up-date of several previous reviews in this area, which again is of great practical importance. The book ends with a short discussion of new polymeric systems which have potential application in solar-energy conversion and storage.

This book gives a well-balanced and up-to-date picture of the various topics of current interest in polymer photochemistry. It contains a wealth of well-presented information about current research, and will be a valuable textbook for academics, industrialists and graduate students. Taken as a whole, it is also a most useful work of reference that will be an essential addition to any library serving those working on polymers. □

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### New editions

- *Deterministic Chaos: An Introduction*, 2nd edn, by H. G. Schuster, with new sections on sensitive parameter dependence, fat fractals and the Farey tree. Publisher is VCH, price is DM108. For review see *Nature* **318**, 241 (1985).
- *Magnetic Resonance Imaging: Basic Principles*, 2nd edn, by Stuart W. Young. Additions include new developments in superconductivity. Publisher is Raven, price is \$49. For review see *Nature* **310**, 803 (1984).



*Electric art — this attractive arrangement of thin sections through electrical cord is taken from the new paperback edition of The Secret House by David Bodanis. The book, a popular, illustrated account of the "unexpected world in which we spend our nights and days", is published by Touchstone, New York, price \$9.95.*

Jan Hirsch/SPL

## Southern pedology

Fiorenzo C. Ugolini

**Antarctica: Soils, Weathering Processes and Environment.** By I.B. Campbell and G.G.C. Claridge. Elsevier:1987. Pp.368. Dfl.220.

CAMPBELL and Claridge must be congratulated on having taken on the onerous task of writing about all the aspects of Antarctic natural systems themselves, rather than procuring an array of experts to produce individual chapters. The result is a well-integrated, readable book in which climate and geological and biological processes are all made pertinent to studies of soils, and which provides a comprehensive view of the Antarctic ecosystem. The authors write fluently and have assembled their material with skill, but the success of the book largely stems from their deep knowledge of the subject.

Following a thorough treatment of geology and geomorphology, climatic zones are defined and related to important determinants of soil properties such as temperature and moisture. Climatic zonation is the basis for distinguishing the main soil moisture regimes — ultraxerous, xerous, oceanic subxerous and moist — and these form the basis for the principal groupings in the soil classification system proposed by the authors. The rest of the chapters are devoted to biology, weathering (physical and chemical), soils and soil properties, soil distribution, salts, soil development and glacial history. There is, too, an appropriate and timely discussion of soils and human activities.

Although physical weathering is recognized as the most important process for rock disintegration, not enough emphasis is placed on the role of unfrozen water in frozen soils and no mention is made of modern theories dealing with this unique state of water. Salt weathering and weathering induced by unfrozen water can explain most of the alteration phenomena

observed in continental Antarctica.

The number of observation and profile descriptions provided is impressive. Systematically presented, these observations have allowed Campbell and Claridge to provide a soil chrono-sequence that reaches into the mid-Miocene, and may prove to be a very valuable tool for reconstructing the fluctuations of the Antarctic ice-sheets. Such a long soil-time relationship is perhaps only possible in Antarctica, where disturbances, so common in warmer regions, are minimized. Thus time becomes the principal factor responsible for soil changes.

Because continental Antarctica is extremely arid, salts tend to accumulate in the soils, and their origin has long been a controversial issue. Weathering, oceanic air masses, marine invasion, sedimentary rocks and auroral activity have all, at one time or another, been suggested as the cause or source. Campbell and Claridge have been able to discriminate between them and present a plausible picture for salt distribution and composition.

Although there are only a few typographical errors, there are one or two other mistakes or slips. Figure 6.3 (p. 127) is printed upside down, perhaps to remind us that this is the way the world is seen from Antarctica. Also, important references are missing in the discussion of ornithogenic soils.

Much of our understanding of soil processes in the cold desert of Antarctica stems from the work of pedologists in the late 1950s and the 1960s. This book does not, perhaps, represent a great advance in knowledge as such beyond those early observations. But Campbell and Claridge have applied the initial concepts beyond the original local studies and have expanded them into a continental view. Their book is a masterly survey of the fundamental principles of Antarctic pedology, and represents the culmination of an era of exploration and scientific work. □

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# Changing concepts of chance

I.J. Good

**The Probabilistic Revolution. Vol.1 Ideas in History.** Edited by Lorenz Krüger, Lorraine J. Daston and Michael Heidelberger. **Vol.2 Ideas in the Sciences.** Edited by Lorenz Krüger, Gerd Gigerenzer and Mary S. Morgan. *MIT Press:1987. Vol.1 pp.449, \$32.50, £29.25. Vol.2 pp.459, \$32.50, £29.25. Set price \$60, £53.95.*

IN 1982–1983 an international group of scholars assembled for about a year in Germany, augmented by visitors, to discuss the events that “made probability a part of philosophy, scientific theories and practice, social policy, and daily life between circa 1800 and 1950” (but mostly before 1930). These meetings led to four first-class books, one by Stephen Stigler (*The History of Statistics: The Measurement of Uncertainty Before 1900*), one by Theodore Porter (*The Rise of Statistical Thinking, 1820–1900*) and the two books now under review. The nationalities of the authors are American 12, German 7, French 4, British 3, Canadian 3, Hungarian 1 and Finnish 1, but all the chapters are written in English.

There are two kinds of history, depending on whether the historian looks at the events through modern eyes or tries to adopt the point of view common at the period discussed. Both approaches are used in these volumes. Volume 1 concentrates mainly on the nineteenth century, and especially on historical, philosophical and social aspects of probability and statistics, while Vol. 2 deals more with the period 1870–1930 and with the great influence of probabilistic thinking on modern psychology, sociology, physiology, evolutionary biology and physics. Probabilistic thinking entered economic thinking in a big way mainly after 1940.

It is difficult to give a succinct picture of a work of some half-a-million words, 35 chapters and 31 authors. One way is to mention statistics *about* rather than *in* the books, beginning with a Hall of Fame which will perhaps convey some impression of the contents.

The following list gives the number of pages on which each of 18 stars is mentioned: Laplace 90, Quetelet 81, R.A. Fisher 59, Darwin 51, Fechner 43, Dobzhansky 38, Sewall Wright 37, Boltzmann 31, Poisson 30, Egon Brunswik 24, Maxwell 24, Wilhelm Wundt 24, Richard von Mises 23, Galton 22, Niels Bohr 21, Cournot 20, Einstein 20 and Gustav Radicke 20. The numbers of people mentioned respectively on  $r$  pages ( $r = 1, 2, \dots, 19$ ) are 391, 113, 65, 40, 29, 18, 15, 15, 10, 11, 4, 2, 2, 5, 5, 5, 3, 4 and 2, fitting

well to the familiar formula  $S/[r(r+1)]$  where  $S = 757$  is the total number of people mentioned. (This formula often fits biological samples and samples of vocabulary, apart from large values of  $r$ . For most applications of the formula only small values of  $r$  matter.)

The total number of mentions is about 2,788. It follows that the approximate coverage of relevant personalities in the work (that is, in further work on the same topics, the probability that the next name to be mentioned will already have been mentioned) is about  $1 - 391/2,788 = 84$  per cent. The expected number of new names in another work of the same size is  $S \log_e(4/e) = 292$ . So there is still scope for further historical research, but diminishing returns have set in, and it would now be more useful to concentrate on the period 1930 to 1980.

Let me now turn to a non-random sample of specifics. Some of the chapters are directly related to the title of the work and tend to justify it. There has been a philosophical swing from Laplacean determinism to quantum mechanical indeterminism, and a revolution from the epistemic interpretation of probability (as depending on our ignorance) to the interpretation of probability in terms of long-run relative frequency; but the latter revolution has revolved past  $180^\circ$  and is now at about  $270^\circ$ . The epistemic interpretation, thought by some to be dead, refuses to lie down.

Even if the world is physically deterministic, in a somewhat metaphysical sense, from a practical point of view it is indeterministic and we don't need quantum mechanics to teach us that. The analogy with pseudorandom numbers is pertinent in this connection and this analogy is useful when discussing irreversibility in classical statistical mechanics. A single ‘chance’ switch in the dealing out of genes leads to the birth of a distinct person. We are all the products of blind chance. Anyone over 60 can reasonably claim, with seeming illusions of grandeur, that if his or her actions had been different when young none of the people now under 20 would ever have been born.

Hacking says

The marvels of modern medicine produce modest increases in life expectancy ... compared to that ... provided by the sanitary movement and its band of ... statisticians. Most people like myself, whose ancestors lived in an urban slum sometime in the nineteenth century, would simply not *exist*, had there not been the sanitary movement. Our ancestors would have died before breeding.

He omits to add that we are all products of chance anyway.

There are three chapters on scientific revolutions and paradigms. For example, Hacking says “*Don't look for a big revolution until you find new kinds of institution that epitomize the new directions created*

*by the revolution*” (his italics). Another sufficient criterion for a revolution is when an early article in a field is originally rejected and is later regarded as pioneering.

None of the authors states the historical hypothesis that paradigms form a rough hierarchy, with those at the top tending to last longer than the sub-paradigms lower down. (For the interpretations of ‘paradigm’ see Margaret Masterman's article in *Criticism and the Growth of Knowledge* Vol.II.) This hypothesis, even if vague or false, suggests a programme of historical and philosophical research.

I was especially struck by Stephen Stigler's chapter in Vol.1, partly because of its brevity and clarity, and because it contains a joke (the only other suspicion of a joke that I noticed was Ian Hacking's use of the lovely word ‘probabilification’). Stigler says

I ... can count on the fingers of one hand (indeed, almost upon the thumbs of one hand) the application of probability assessments of accuracy in the social sciences before 1880. ... Together, they [Francis Galton, Francis Edgeworth, and Karl Pearson] helped launch a statistical revolution in social science akin to that which Laplace had launched in astronomy.

Swijtink mentions R.A. Fisher's argument that distinguishes between deductive and inductive thinking. In deductive thinking a conclusion can be reached by using only part of the evidence, but this (in principle) is unjustifiable in inductive thinking. Paradoxically, the precision of the results obtained by the ‘anti-Bayesians’ can be obtained only by ignoring some of the evidence, especially in distribution-free techniques in statistics.

In Vol.2, Mary Morgan says “... probability ideas have been used successfully to plug one gap in the system of economic theory only to find that uncertainty [in Frank Knight's sense, 1921] remains as considerable as ever”. Another way to express this, that some of us would prefer, is to say that subjective probabilities do not typically have sharp values, they are interval-valued. This important aspect of epistemic probability is, I think, not mentioned anywhere in these two volumes.

M.J.S. Hodge discusses explanation in natural selection and mentions that Darwin “conformed his argument to the *vera causa* ideal for a scientific theory”. A *vera causa* is an explanation “known to exist ... and not merely conjectured to exist”. My own concept of ‘explicativity’ is more general because it allows for the probability of an explanatory hypothesis (for example, see a chapter in my book *Good Thinking*).

I found the last chapter, by Nancy Cartwright, especially interesting. She says that the basic attitude of American physicists in the 1920s and 1930s, as contrasted with that of some of their European counterparts, was that “the task of



physics is not to explain but to describe" and she quotes Kemble (1929): "... we may address ourselves to the task of describing what we observed in the most compact manner possible". The question arises why compactness of description is desirable. Is it because it saves expense, or is it because a shorter description is, after all, more likely to lead to a good explanation and to get us closer to an understanding of the world? It would be a great blow to natural selection if its explanatory power were to be disregarded, so it seems that the views of most biologists about the purposes of theorizing must differ very much from those of most American physicists, those in the 1920s and 1930s at least. Cartwright says that her aim is "to document the lack of philosophical perplexity [among physicists]; ... [and] to argue that it was entirely rational". It is similarly entirely rational for bookbinders to ignore the chemistry of gum and to be more concerned, for example, in making books that lie flat. Unfortunately the binders of these books have failed in this respect.

Cartwright says "To ask what happens when observations cannot be made is a meaningless question, ...". I don't know her attitude to virtual particles and to quarks but I have not yet read her book on laws of nature.

The true history of probability, or of science in general, will never be written because so much depends on unrecorded oral communication, and also because writers often do not cite their sources. So all history is to some extent misleading but these two volumes come close to the best that can be achieved on the topics and the period covered. They contain a mine of historical and philosophical material. You can dip in almost anywhere, and be interested, whether you agree or disagree with what is said. Learning about the historical roots of your discipline has much the same kind of interest as learning about your own ancestral roots. □

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### Spring books

Nature's next book review supplement, Spring Books, appears in the issue of 21 April.

Among the titles to be reviewed are *Infinite in all Directions* by Freeman Dyson, two on science in the Antarctic, and two others on the history of the Lick Observatory (which celebrates its centenary this year), Virginia White's *Handbook of Research Laboratory Management*, *Smallpox and its Eradication* edited by F. Fenner and colleagues, *H.A. Kramers* by Max Dresden and *Eye, Brain, and Vision* by David H. Hubel.

Among the reviewers are John Stachel, Desmond King-Hele, Nicholas Kemmer, Warren A. Sinclair and R.W. Guillery.

## Chaos illumined

Graham Dunn

**Chaos in Biological Systems.** Edited by H. Degn, A.V. Holden and L.F. Olsen. Plenum:1987. Pp.323. \$62.50 (North America, \$75 (elsewhere)).

MANY biologists are deeply sceptical about the merits of applying advanced mathematical reasoning to biological problems. Until recently, this attitude was largely justified. A leading mathematician and cell biologist once told me that, of four possible categories in the application of mathematics to biology, only one is missing: good mathematics applied to good biology. Even so, mathematical methods have proved essential for the development of biometry as an objective science, and many other applications of mathematics to biology have been far from trivial; the morphogenetic theories of Turing and Thom, for example, may yet become respectable candidates for the missing category. But sophisticated mathematical reasoning has not had a widespread effect, so far, on our understanding of biological processes.

*Chaos in Biological Systems* may be among the heralds of a dramatic change. Here chaos is illumined, not by flashes of lightning as in the words of Oscar Wilde, but by mathematics. Chaos theory, a topic in the forefront of research into non-linear dynamic systems, is now promising to have profound implications throughout biology and medicine as well as in the physical, chemical and socio-economic sciences. The name of the theory is little more than a decade old although, together with Turing's and Thom's ideas, the roots of chaos theory in mathematics go back at least as far as Poincaré's theory of bifurcations in the late nineteenth century. Turing's pioneering computer programs of 1951 were non-linear models of biological morphogenesis, and it is the recent development of fast and cheap computing that has now enabled mathematicians to begin experimenting freely with non-linear systems of equations.

The hope for biology is that fairly simple non-linear systems may be able to represent the regulatory feedback loops that lie at the core of practically all biological dynamics from molecular interactions to predator-prey relations. Many such systems of equations can be found in the book, and the behaviour of these models is often quite lifelike. Striking spatial patterns can emerge from homogeneous starting conditions and complex periodic behaviour, or even random fluctuations can develop in simple systems — hence the name 'chaos'.

The book is a collection of papers presented at a NATO Advanced Research

Workshop held in Cardiff in December 1986. The first impression on scanning the list of titles is of the staggering range of biological systems that are now thought to show chaotic dynamics. Among the topics represented are pathological heart rhythms, plant transpiration, platelet regulation, receptor binding, enzyme dynamics, slime mould aggregation, morphogenesis, ecology, disease epidemics and population growth, and a large section is devoted to neuronal dynamics. Not all the papers conclude that the dynamics of the system under study are chaotic, but it seems that no level of biological organization can be excluded from chaotic behaviour.

Even so, the book does not exhaustively cover the areas of current interest, and the more involved but more exciting topics, such as control of gene expression, embryogenesis and evolution, are scarcely mentioned. Nor is the book intended as a primer for biologists. Some of the mathematics is heavy going even for enthusiasts. Nevertheless, many biologists will find browsing through the book to be a rewarding experience, though it is a pity that no general introduction or glossary of the whimsical jargon of chaos theory is included; strange attractors, renormalization group algorithms and subharmonic bifurcations all appear on page 1.

Many, if not most, of the main problems in contemporary biology are to do with the dynamics of interactions. As with the famous chemical reaction discovered by Belousov and Zhabotinsky, the rules of interactive behaviour are sometimes fairly well understood but the behaviour that emerges is often unexpectedly complex. What this book shows is that chaotic dynamics are probably widespread in biological systems. If this is true, chaos theory denies the hope that the behaviour of such systems will ever be deducible or predictable from a knowledge of the rules and so the cherished stratagem of biologists, molecular reductionism, may prove ineffective in unravelling these problems. But chaos theory offers the new hope, hardly tenable before, that the underlying dynamics of complex biological behaviour may often be relatively simple and hence understandable.

To show that a biological system can behave chaotically may not seem to say a lot about it — at least to outsiders — but these are early results that might be comparable to the early results in biochemistry of the nineteenth century. As I write, sitting in front of a chart of biochemical pathways, I wonder whether biomathematicians of the next century will emulate the magnificent achievements of twentieth-century molecular reductionists. □

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# The influence of supernovae and passing stars on comets in the Oort cloud

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*The heating of Oort cloud comets by supernovae and passing stars dominates their thermal history and may provide a source of dust and refractory heavy elements to the interstellar medium.*

THE dynamical evolution of the Oort cloud has been tied intimately to the nearby passage of stars and large molecular clouds<sup>1</sup>. It has been established<sup>2</sup> that such encounters randomize comet orbits, deplete the cloud population, and induce 'comet showers' in the inner solar system. In this paper, we examine the thermal effects induced by supernovae and passing stars. Our main goals are: (1) to determine whether Oort cloud comets remain fully pristine after these encounters; and (2) to assess the possible feedback of dust and refractory heavy elements from the Oort cloud to the interstellar medium.

Some  $10^{12}$  comets are thought to reside in an outer Oort cloud of radius  $R_2 \approx 10^5$  AU surrounding the Sun<sup>3</sup>. To replenish these comets against dynamical loss mechanisms, investigators have postulated<sup>4,5</sup> the existence of an inner Oort cloud of radius  $R_1 \approx 10^4$  AU containing up to  $10^{14}$  comets. Comets are thought to have masses  $\sim 10^{16}$ – $10^{17}$  g, and the Solar System's cometary reservoir may total  $\sim 10^{30}$  g.

To heat a comet surface element at an angle of incidence  $\theta$  to a blackbody temperature  $T_e$  in the absence of other radiation sources, an object of luminosity  $L_*$  must lie within a radius of influence,

$$R_i(T_e) = \left[ \frac{L_* \cos \theta}{4\pi\sigma_{SB}T_e^4} \right]^{1/2} \approx (0.84 \text{ pc}) \left[ \frac{L_* \cos \theta}{10^6 L_\odot} \right]^{1/2} T_{30}^{-2} \quad (1)$$

where  $T_{30} = (T_e/30 \text{ K})$ ,  $1 \text{ pc} = 3.086 \times 10^{18} \text{ cm}$ , and  $L_\odot = 3.89 \times 10^{33} \text{ erg s}^{-1}$ . For the global average comet temperature,  $R_i$  is a factor of two smaller than the maximum (at  $\theta = 0$ ). Using this simplified model for a star of  $1 L_\odot$ , we find that the 30-K subsolar-point radius of influence is 173 AU.

To evaluate the heating of comets by passing stars of luminosity  $L_*$ , we first obtain an expression for the volume of space within the Oort cloud heated above a specified temperature  $T_e$ . We then obtain an expression for the cumulative effect of many such encounters, summing over stellar types in proportion to their observed density.

To accomplish our goals, we compute the total fraction of Oort cloud comets processed to temperature  $T_e$  by passing stars,

$$f_p(T_e) = \sum_i \frac{N_{e,i}(T_e) V_{p,i}(T_e)}{(4\pi R_2^3/3)} \quad (2)$$

where  $N_{e,i}$  and  $V_{p,i}$  are, respectively, the number of encounters and the average volume of the Oort cloud processed to temperature  $T_e$ , as computed below. Notice that although  $V_{p,i}$  cannot exceed  $(4\pi R_2^3/3)$  in any one encounter,  $f_p > 1$  is possible once the fractional effects of many encounters are summed. The interpretation would then be that the Oort cloud has been processed to  $T_e$  more than once. We assume that the Oort cloud is spherical, with comets distributed homogeneously. By evaluating the fractional volume processed, we avoid any assumptions about the total number of comets.

The average volume of the Oort cloud processed to tem-

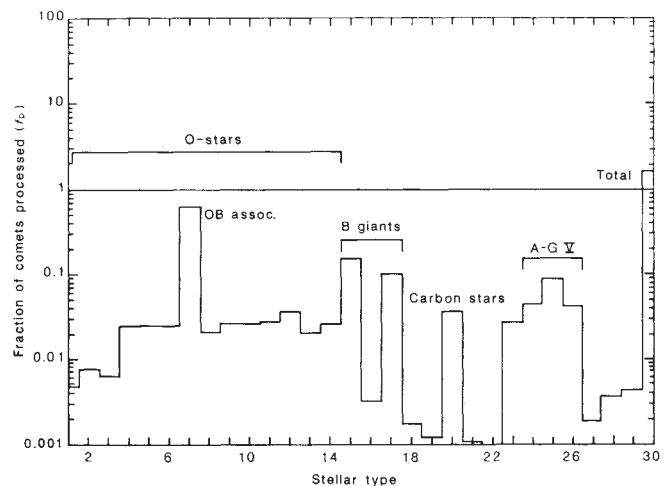
perature  $T_e$  by a single random encounter with  $R_i < R_2$  may be approximated as

$$V_{p,i} = \pi R_i^2 \bar{R}_{\text{enc}} \approx \pi R_i^2 [\sqrt{2} R_2] \quad (3)$$

where  $\bar{R}_{\text{enc}}$  is the median path length of encounters through a spherical Oort cloud. If we envisage an encounter of a star with the Oort cloud as 'carving out' a tunnel in which comets have been heated, then  $R_i$  corresponds to the radius of the 'bore hole'.

Equation (3) holds because encounters between the Sun and a star of substantial mass ( $40$ – $100 M_\odot$ ) at a distance comparable to  $R_i \approx 10^5$  AU may be approximated by a rectilinear path, and because gravitational focusing of comets into the volume of influence  $V_{p,i}$  has a negligible effect on the fraction of comets processed. One can also show that  $R_i$  for luminous stars greatly exceeds the gravitational radius of influence. Thus an insignificant fraction of heated comets will be subjected to appreciable gravitational perturbations which might eject them from the cloud.

Bolometric luminosities are used throughout this study, in which we consider two classes of heating events: (1) encounters with passing luminous stars; and (2) stochastic (stationary) events such as novae and supernovae. The distinction between the two classes lies in the lifetime of the source—that is, whether  $R_i$  is large or small compared with the distance moved during the source lifetime.



**Fig. 1** Fraction  $f_p$  of Oort comets heated to  $T_e \geq 16$  K by passing stars of 29 types (Table 1). Column 30 is the sum over all stellar types; supernova heating is not included. Columns with no data indicate  $f_p \leq 10^{-3}$ , but are included in the sum. O-type stars are the dominant heating source, with OB associations being the most influential single type. Overall, passing stars should process every comet in the cloud at least once to 16 K.



In case (1), the total number of encounters of the Oort cloud with stars over the age of the solar system,  $\tau \approx 4.5 \times 10^9$  yr, is given by

$$N_e = \tau \sum_i n_i \sigma_i \langle v_{c,i} \rangle \quad (4)$$

where  $n_i$  is the space density of stars,  $\sigma_i = \max(\pi R_2^2, \pi R_1^2)$  is the encounter cross-section, and  $\langle v_{c,i} \rangle$  is the mean collision velocity between the Sun and stars in class  $i$ , with three-dimensional velocity dispersion  $v_i$ , assuming a solar motion  $V_0 \approx 20$  km s<sup>-1</sup>. Throughout, we shall assume  $n_i$  and  $v_i$  are time-invariant<sup>6</sup>. Because of our definition of  $\sigma_i$ , equation (3) includes those encounters in which the interloping star physically misses the Oort cloud, but the star's radius of influence extends into the cloud, causing a fraction of the comets to be heated.

In case (2), the lifetime of the source is sufficiently short (less than a year) that the number of heating events is set by the probability of overlap with stationary sources within the distance  $R_i$ ,

$$N_e = \tau \sum_i \xi_i \frac{4\pi R_i^3}{3} \quad (5)$$

where  $\xi_i$  is the rate of events per unit volume per unit time and  $R_i$  is the appropriate radius of influence.

### Passing stars

To calculate  $f_p$  for passing stars, we first obtain an improved form for the radius of influence,  $R_i$ . To determine accurately an equilibrium temperature,  $T_e$ , one must go beyond the rough formulation in equation (1) and include all sources of incident radiation. The ambient temperature of an Oort cloud comet in the absence of a passing luminous star is 4.4 K, assuming solar, mean interstellar, and 2.73 K background radiation. We define the radius of influence,  $R_i$ , as the distance to which a star of luminosity  $L_*$  will heat a body of albedo  $a$  and emissivity  $\epsilon$  to temperature  $T_e$ . We conservatively assume  $a = 0.05$  and  $\epsilon = 0.8$ . Because vapour pressures over ice depend exponentially on temperature, the temperature of the sub-stellar point (that is, the hottest point on the comet during an encounter) is the critical parameter in determining the degree of cometary heating.

We will later demonstrate that encounter timescales are much longer than comet rotation timescales. In a random encounter with a luminous star, the planes of comet rotation and comet-star relative motion will not be aligned, and a large fraction of the comet's surface will spend a substantial amount of time near the sub-stellar point ( $\cos \theta = 1$  in equation 1). Thus, we choose  $T_e$  to represent the temperature of the sub-stellar point, which radiates into  $\sim \pi$  steradians.

For a comet at a distance  $R_c$  from the Sun ( $R_c < R_2$ ) under the influence of solar, stellar and background radiation sources, the radius of influence is

$$R_i = \left[ \frac{L_*/4\pi}{\epsilon \sigma_{SB} T_e^4 (1-a) - F_b} \right]^{1/2} \quad (6)$$

where  $\sigma_{SB}$  is the Stefan-Boltzmann constant and where

$$F_b = F_{\text{sun}} + F_{\text{CMB}} + F_{\text{star}} + F_{\text{OBA}} \quad (7)$$

represents the background flux (erg cm<sup>-2</sup> s<sup>-1</sup>) from the Sun, cosmic microwave background, ambient starlight, and radiation in OB associations. We adopt  $F_{\text{sun}} = L_{\odot}/4\pi R_c^2$ ,  $F_{\text{CMB}} = 3 \times 10^{-3}$  erg cm<sup>-2</sup> s<sup>-1</sup>,  $F_{\text{star}} = 5 \times 10^{-3}$  erg cm<sup>-2</sup> s<sup>-1</sup>, and  $F_{\text{OBA}} = (3N_* L_*/4\pi R_{\text{OB}}^2)$  for an OB association of radius  $R_{\text{OB}}$  containing  $N_*$  O-type stars of luminosity  $L_*$ .

As we will later demonstrate, luminous O- and B-type stars and red supergiants dominate the heating. From a catalogue of galactic O-stars (C. D. Garmany, unpublished data), we estimate the local surface density of all O-type stars to be  $\sim 30$  kpc<sup>-2</sup>, equivalent to a spatial density  $n \approx 3 \times 10^{-7}$  pc<sup>-3</sup> in a disk of total thickness 100 pc. Humphreys and McElroy<sup>7</sup> show that the B-supergiants have a surface density  $\sim 53$  kpc<sup>-2</sup> (for magnitude

Table 1 Stellar population data

| Type | Stellar type*  | log $n_i$ | log ( $L_i/L_{\odot}$ ) | $v_i$ (km s <sup>-1</sup> ) | log $R_{i,16}$ (AU)† |
|------|----------------|-----------|-------------------------|-----------------------------|----------------------|
| 1    | O3-4 III       | -9.2      | 6.0                     | 17                          | 5.88                 |
| 2    | O3-4 I         | -9.0      | 6.0                     | 17                          | 5.88                 |
| 3    | O3-4 V         | -8.9      | 5.8                     | 17                          | 5.78                 |
| 4    | O5-6.5 I       | -8.3      | 5.8                     | 17                          | 5.78                 |
| 5    | O7-8.5 I       | -8.2      | 5.7                     | 17                          | 5.73                 |
| 6    | O5-6.5 III     | -8.2      | 5.7                     | 17                          | 5.73                 |
| 7    | OB assoc.      | -6.8      | —                       | 17                          | —                    |
| 8    | O7-8.5 III     | -8.0      | 5.4                     | 17                          | 5.58                 |
| 9    | O9-9.5 I       | -7.9      | 5.4                     | 17                          | 5.58                 |
| 10   | O5-6.5 V       | -7.9      | 5.4                     | 17                          | 5.58                 |
| 11   | O9-9.5 III     | -7.7      | 5.2                     | 17                          | 5.48                 |
| 12   | O7-8.5 V       | -7.5      | 5.1                     | 17                          | 5.43                 |
| 13   | Wolf-Rayets    | -8.1      | 5.0                     | 17                          | 5.63                 |
| 14   | O9-9.5 V       | -7.4      | 4.8                     | 17                          | 5.28                 |
| 15   | Early B giants | -6.4      | 4.7                     | 19                          | 5.18                 |
| 16   | M0 I           | -7.9      | 4.5                     | 17                          | 5.08                 |
| 17   | Late B giants  | -6.3      | 4.3                     | 19                          | 4.98                 |
| 18   | K5 I           | -7.9      | 4.1                     | 17                          | 4.88                 |
| 19   | A5 I           | -8.0      | 4.0                     | 19                          | 4.83                 |
| 20   | Carbon stars   | -7.0      | 4.0                     | 56                          | 4.83                 |
| 21   | F5 I           | -8.0      | 3.9                     | 17                          | 4.78                 |
| 22   | G5 I           | -8.0      | 3.8                     | 17                          | 4.73                 |
| 23   | Miras          | -6.6      | 4.0                     | 50                          | 4.58                 |
| 24   | A5 V           | -3.3      | 1.2                     | 24                          | 3.43                 |
| 25   | F5 V           | -2.6      | 0.6                     | 36                          | 3.13                 |
| 26   | G5 V           | -2.2      | -0.1                    | 39                          | 2.78                 |
| 27   | K5 V           | -2.0      | -0.8                    | 43                          | 1.98                 |
| 28   | White dwarfs   | -1.6      | -2.0                    | 63                          | 1.83                 |
| 29   | M5 V           | -1.2      | -2.1                    | 42                          | 1.78                 |

Adopted spatial densities  $n_i$  (pc<sup>-3</sup>) and luminosities  $L_i$  of stellar types: OB and Wolf-Rayet stars (from refs 7, 40 and Garmany catalogue); Miras and carbon stars<sup>8-11</sup>; other stars<sup>6</sup>. Root mean square velocities  $v_i$  are from ref. 6; solar motion  $V_0 = 20$  km s<sup>-1</sup>.

\* O-star populations are divided equally between field stars and OB associations ( $N_* = 10$  O-stars per association, each star with mean luminosity  $10^{5.8} L_{\odot}$  after accounting for binaries). The O-star counts from the Garmany catalogue are multiplied by an incompleteness factor of 1.2 and agree with totals counts from Humphreys and McElroy<sup>7</sup>.

† Logarithm of radius of influence (in AU) for  $T_e = 16$  K, albedo  $a = 0.05$ , emissivity  $\epsilon = 0.8$ , and additional background sources (see equation 7).

bins  $M_{\text{bol}} = -7$  and  $-6$ ), although their survey may be incomplete. The surface density of carbon stars ( $\sim 10^4 L_{\odot}$ ) has been estimated<sup>8,9</sup> at  $\sim 40$  kpc<sup>-2</sup>, that of the asymptotic giant S-stars<sup>10</sup> ( $\sim 10^4 L_{\odot}$ ) at  $\sim 13$  kpc<sup>-2</sup>, and that of the short-period Mira variables<sup>11</sup> ( $\sim 3,000 L_{\odot}$ ) at  $\sim 400$  kpc<sup>-2</sup>. (The long-period Miras have somewhat lower surface density, but comparable luminosity.) Table 1 summarizes our adopted luminosities and space densities. These data agree well with similar compilations by Miller and Scalo<sup>12</sup>.

At distances greater than  $10^5$  AU, luminous O-stars and red supergiants can raise a comet to significant temperatures ( $T \approx 40$  K). In contrast, A, F, G and K main sequence stars heat only a pencil-thin tunnel, 10–100 AU across, as they pass through the Oort cloud. Because O-stars are a critical element in stellar heating, we have further refined our calculation in three ways. First, we include runaway stars<sup>13</sup> as a 20% population with mean velocity 40 km s<sup>-1</sup>. Second, we correct the O-star luminosities in the Garmany survey for the probable fraction ( $\sim 40\%$ ) which are multiple systems<sup>14</sup> of which  $\sim 85\%$  contain another O-star<sup>15</sup>. Finally, we have divided O-type stars equally into 'field stars', and 'associations' with a mean content of 10 O-type stars and a 50 pc mean radius<sup>16</sup>. Encounters with OB associations elevate the radiation field,  $F_b$  in equation (7), during the association encounter.

Figure 1 depicts the fractional volume of the Oort cloud processed over the age of the Solar System for each type of star

in our data set (Table 1). The OB associations dominate the stellar heating, because they contain half the O-stars and because of the elevated radiation field during an encounter. Altogether, we estimate that  $>10^4$  stars have heated some fraction of the Oort cloud over the age of the Solar System, in agreement with dynamical studies<sup>17</sup>.

Figure 2 shows the fraction of Oort comets which have been processed to various temperatures by passing stars. From these data, we find that every comet in the Oort cloud is heated to 16 K at least once by passing stars, 50% have been heated to 22 K, and 10% have reached 34 K. Essentially all of this heating results from encounters with luminous stars.

There are several aspects in which our model is conservative and omits effects which could increase the net heating by passing stars. One such effect is the larger ( $T \sim 5$  K) cosmological background temperature  $4.5 \times 10^9$  years ago<sup>18</sup>; including this would result in a 20% time-averaged enhancement in  $f_p$ . Second, we have used OB star densities characteristic of the local 2 kpc, which may be atypical of the Galaxy as a whole. Finally, the population of luminous stars may have been significantly higher at various times in the past. Models of stellar populations and colours<sup>12,19</sup> suggest that star formation is irregular on  $10^8$ -yr timescales and may have been up to a factor of five higher in the past. If so, the degree of comet heating would be increased, depending on the extent to which luminous stars escaped the gas-rich spiral arms.

## Novae and supernovae

Whereas the most luminous stars have luminosities  $\sim 10^6$  times that of the Sun, the brightest 'stellar objects' are novae and supernovae. For a few months, a supernova can reach luminosities in excess of  $10^9 L_\odot$ , releasing  $\sim 10^{49}$  erg in light,  $\sim 10^{51}$  erg in mechanical (blast-wave) energy and  $\sim 10^{53}$  erg in neutrinos<sup>20,21</sup>. We concentrate here on the electromagnetic luminosities of supernovae. Although novae are  $\sim 10^3$  times more frequent than supernovae<sup>22</sup>, their luminosities are  $\sim 10^4$  times lower and thus their thermal effects are approximately 10% those of supernovae.

Supernovae come in two general types<sup>23</sup>, reflecting their spectral features, their location in the Galaxy, and their progenitor stars. Type I supernovae are believed to arise from the explosion of a white dwarf star undergoing rapid mass transfer in a close binary, and occur in an older disk and bulge population. Type II supernovae are thought to result from the core collapse and rebound which ends the life of a massive star in the galactic disk or spiral arms.

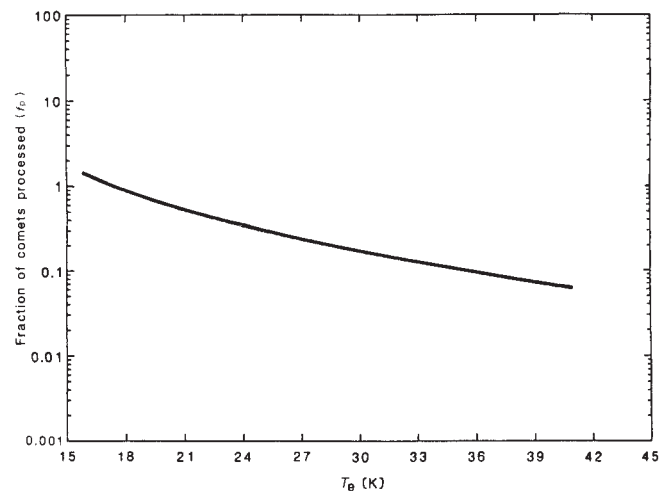
For our calculations, we shall require the peak supernova luminosities  $L_{SN}$  and formation rates  $\xi_{SN}$ . Tammann<sup>23</sup> has reviewed the statistical properties of supernovae, based on a combination of studies of external galaxies and historical supernovae in the Milky Way. The peak magnitudes of supernovae contain intrinsic dispersion about their means, and an uncertainty due to the adopted cosmological distance scale based on the Hubble constant  $H_0 = (100 \text{ km s}^{-1} \text{ Mpc}^{-1})h$ , where  $h$  is a scaling parameter in the range 0.5–1.0. Tammann finds that the mean peak absolute B-magnitudes of supernovae are  $-19.7 \pm 0.5$  (Type I) and  $-19.0 \pm 1.6$  (Type II) on the distance scale in which  $h = 0.5$ . Including a 0.15 magnitude bolometric correction, these magnitudes correspond to total luminosities of  $L(\text{Type I}) = (1.7 \times 10^9 L_\odot)h^{-2}$  and  $L(\text{Type II}) = (0.9 \times 10^9 L_\odot)h^{-2}$ . The radii of influence (equation 1) are then

$$R_1(\text{Type I}) = (17.3 \text{ pc}) T_{30}^{-2} h^{-1} \quad (8)$$

$$R_1(\text{Type II}) = (12.7 \text{ pc}) T_{30}^{-2} h^{-1} \quad (9)$$

Clearly, supernovae can heat comets from very large distances. Since  $R_1 \gg R_2$ , the entire Oort cloud is heated uniformly ( $T_e < 100$  K) by a supernova. Therefore, the fraction of the Oort cloud processed reduces to the number of encounters,  $N_e$ .

The supernova rates,  $\xi_{SN}$ , are also somewhat uncertain. Tammann<sup>23</sup> estimates mean intervals of 36 yr (Type I) and 44 yr



**Fig. 2** Fraction of comets heated by all types of passing stars versus equilibrium temperature  $T_e$  during encounters. Notice that all comets have been heated by passing stars to  $\sim 16$  K, while at least 10% should have been heated to  $\sim 34$  K. Supernova heating of Oort cloud comets is not included.

(Type II) in the Galaxy, for a total of one supernova every 20 yr. Estimates from pulsar counts<sup>24</sup> suggest one pulsar formed every 30–120 yr (presumably from a Type II supernova), with a best estimate of  $56 \pm 9$  yr (ref. 25), depending on observational uncertainties. Estimates from observed radio supernova remnants<sup>26</sup> suggest an interval of 40–150 yr (many of these remnants may be missed if the supernovae explode in a hot, low-density phase of interstellar matter). If we adopt the Tammann rates and assume a galactic disk area of  $830 \text{ kpc}^2$  and vertical scale-height (half thickness) of 300 pc (Type I) and 100 pc (Type II), we arrive at rates  $\xi_{SN} = 0.54 \times 10^{-13} \text{ pc}^{-3} \text{ yr}^{-1}$  (Type I) and  $1.4 \times 10^{-13} \text{ pc}^{-3} \text{ yr}^{-1}$  (Type II). Similar values are found by other authors<sup>27,28</sup>. These rates yield the number of encounters that heat comets to a temperature  $T_e = (30 \text{ K}) T_{30}$ ,

$$N_e = 5.3 T_{30}^{-6} h^{-3}; \quad \text{Type I} \quad (10)$$

$$N_e = 5.4 T_{30}^{-6} h^{-3}; \quad \text{Type II} \quad (11)$$

Thus, for  $h = 0.75$ , the number of supernova events is approximately  $N(T_e) \approx 25 T_{30}^{-6}$ . Therefore, all comets have been heated 25 times to  $T_e \approx 30$  K and at least once to  $T_e \approx 50$  K. There is a 50% probability that the Oort cloud has been heated as high as  $\sim 60$  K. Relaxing supernova formation rates<sup>28</sup> by a factor of two (one supernova every 40 years in the galactic disk), one finds that all comets should have had at least one encounter to 45 K.

As OB stars are clustered, it is also highly likely that many of the Type II supernovae occurred during the encounters between the Sun and OB associations discussed above. We estimate that the Solar System has undergone  $\sim 10$  such encounters with OB associations of radius 50 pc, for a total of 40 million years of exposure.

## Discussion

We have demonstrated that the passage of luminous stars through the Oort cloud over the age of the Solar System will subject all comets to heating episodes to at least 16 K, and a sizeable fraction to 30 K. Stochastic supernovae have even more striking effects during month-long events; conservative estimates of the supernovae rate in the galactic disk suggest that most comets have been heated to 45 K, and a fraction to  $\sim 60$  K. These results imply that comets are not fully pristine relics of Solar System formation.

The thermal skin-depth of the comet surface is defined<sup>29</sup> by  $d = (\kappa \tau_h / \pi)^{1/2}$ , where  $\kappa$  is the thermal conductivity coefficient



and  $\tau_h$  is the timescale for heating. An average encounter with a star of luminosity  $L_*$  and velocity  $v_i = 30 \text{ km s}^{-1}$  typically lasts a time

$$t_{\text{enc}} \approx (2^{1/2} R_i / v_i) \approx (4 \times 10^4 \text{ yr}) \left( \frac{L_*}{10^6 L_\odot} \right)^{1/2} T_{30}^{-2} \quad (12)$$

Thus, a G-star encounter lasts  $\sim 10$  yr, while luminous stars cause comets to bask in an elevated radiation field for  $\sim 10^4$  yr, both much longer than typical comet rotation periods of hours to days.

For solid ice,  $\kappa$  is a weakly decreasing function of temperature. For our purposes we approximate  $\kappa$  by  $1.45 \times 10^{-2} \text{ cm}^2 \text{ s}^{-1}$  for crystalline ice. For amorphous ice<sup>30</sup>,  $\kappa$  can be a factor of 10 lower; porosity effects could lower  $\kappa$  by another factor of 10. Using  $\tau_h \approx (\sqrt{2} R_i / v_i)$  for passing stars and taking  $T_e = 16 \text{ K}$ , we estimate that the processing depth  $d \approx 20$ –60 m for passing stars. Taking  $T_e = 45 \text{ K}$  and  $\tau_h \approx 1$  month, we find  $d < 1$  m for supernovae. Thus, supernovae briefly heat a thin surface layer to high temperatures, whereas passing stars heat a much deeper layer, corresponding to  $\sim 6$ –20% of a typical comet mass, to a lower temperature for extended periods.

The heating of comets in the Oort cloud can induce both morphological and abundance changes. These mechanisms can also explain certain observations of 'new comets'. Table 2 presents a list of key temperatures which affect the evolution of cometary surfaces. For encounters with passing stars and supernovae, significant vapour pressures will be achieved for the cosmogonically abundant ices of Ne, Ar, CO, CH<sub>4</sub>, O<sub>2</sub>, N<sub>2</sub> and S<sub>2</sub>. Over the  $10^4$ -yr timescales characteristic of such encounters, we expect these ices to be depleted from the outermost layers of comets, modifying both the mechanical structure and chemical composition of the cometary surface. We note that some new comets are observed<sup>31,32</sup> to demonstrate depletions in the volatile ices N<sub>2</sub>, CO and CH<sub>4</sub> relative to equilibrium condensation scenarios for the outer Solar System; studies of Ne and Ar depletions must await the launch of suitable far-ultraviolet payloads. Volatile depletions directly affect the observability and behaviour of comets at large heliocentric distances.

The passage of luminous OB-stars and red giants through the Oort cloud will also subject cometary surfaces to erosion by strong stellar winds. Stern<sup>33</sup> has shown that high-velocity grain impacts efficiently erode cometary surfaces. This effect will be discussed fully in a subsequent paper; for the present, we estimate that 0.5–5.0 cm of material may be lost from each comet in the Oort cloud to the interstellar medium due to encounters with luminous stars.

If comet clouds are common around solar-type and low-mass stars, then stellar encounters can provide a potentially interesting feedback mechanism between planetary systems and the interstellar gas and grains. A simple estimate shows that Oort-type clouds around cool stars in the solar vicinity (of density  $\sim 0.5 \text{ pc}^{-3}$ ) could be a repository for a mass density equal to 1% that of the interstellar medium. However, cometary dust-to-gas ratios<sup>34,35</sup> are observed to vary between 0.1–1.0 by mass, compared to just 0.006 in the interstellar medium<sup>36</sup>. Thus, although grain liberation by the sublimation of frosts is not important at the low temperatures characteristic of stellar encounters, heavy elements can be liberated by the ejection of ice grains during cometary heating episodes and provide a significant perturbation to interstellar abundances. Such ejection phenomena have been observed in the laboratory<sup>31</sup>. Cometary input of dust grains to the interstellar medium could provide the 'new source' of grains required to understand interstellar grain formation-destruction equilibrium<sup>37,38</sup>. The largest cometary enhancements of interstellar abundances could come for refractory elements such as Si, Mn, Fe, Ca, Ti, and Ni, which are typically depleted<sup>36,39</sup> from the gas phase (onto solid dust particles) by factors of 10–1,000.

We have demonstrated that both supernovae and passing luminous stars can heat, modify, and thus evolve comets in the

**Table 2** Selected critical temperatures for comets

| Temperature (K) | Event                                                                                                        |
|-----------------|--------------------------------------------------------------------------------------------------------------|
| 5               | H <sub>2</sub> sublimation <sup>41</sup>                                                                     |
| 16              | Ne vapour pressure reaches 1 torr                                                                            |
| 17–26           | Trapped H released from H <sub>2</sub> O clathrates <sup>31</sup>                                            |
| 22              | $T_{\text{subl}}$ for N <sub>2</sub> (ref. 31)                                                               |
| 24              | $T_{\text{subl}}$ for O <sub>2</sub> (ref. 41)                                                               |
| 25              | $T_{\text{subl}}$ for CO (ref. 41)                                                                           |
| 25              | All Ne in H <sub>2</sub> O clathrates expelled                                                               |
| 27              | 'Yellow stuff' explosions <sup>42</sup>                                                                      |
| 30              | Onset of S <sub>2</sub> elimination <sup>42</sup>                                                            |
| 31              | CO and Ar turn on <sup>31</sup>                                                                              |
| 31              | $T_{\text{subl}}$ for CH <sub>4</sub> (ref. 41)                                                              |
| 34              | CH <sub>4</sub> turns on <sup>31</sup>                                                                       |
| 40              | Heavy cracking, whitening of H <sub>2</sub> O ice <sup>31</sup>                                              |
| 40              | Rapid evaporation of CH <sub>4</sub> , CO, and micron-sized H <sub>2</sub> O particle ejection <sup>31</sup> |
| 40              | Formation of formaldehyde (H <sub>2</sub> CO) and formamide (NH <sub>2</sub> HCO) <sup>42</sup>              |
| 42              | $T_{\text{subl}}$ for C <sub>2</sub> H <sub>4</sub> (ref. 42)                                                |
| 57              | $T_{\text{subl}}$ for C <sub>2</sub> H <sub>2</sub> , H <sub>2</sub> S (ref. 41)                             |

Oort cloud. We have further demonstrated that such encounters will dominate the thermal history of comets in the cloud. Passing stars and supernovae should produce signatures in the surface composition and microstructure of comets, and can influence the sublimation development of new comets approaching the Sun. Such signatures could be detected by *in situ* cometary studies, or more directly by the return of cometary samples to Earth. Although the probability of a close encounter ( $R_i < 10^5 \text{ AU}$ ) between a luminous star and the Oort cloud is small ( $\sim 2\%$ ), it would cause millions of comets simultaneously to undergo heating to  $T \sim 30 \text{ K}$  and several hundred comets to  $T \sim 300 \text{ K}$ . Such an event would produce prodigious quantities of H<sub>2</sub>O vapour-generated H and OH, and cause other more volatile molecules (and cometary dust) to be liberated. These molecules will fluoresce in accordance with the Swings effect and re-emit Lyman  $\alpha$  and other photons slightly Doppler shifted off the interloper's spectrum by the encounter velocity  $v_i$ . A promising location to see this effect is an O-star within a distance  $R_i(T_e)$  of a main-sequence dwarf. The search for and identification of such signatures could lead to the detection of extra-solar Oort clouds. The detection of extra-solar Oort clouds could guide us toward likely sites in which to search for extra-solar planets.

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# A novel potent vasoconstrictor peptide produced by vascular endothelial cells

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*An endothelium-derived 21-residue vasoconstrictor peptide, endothelin, has been isolated, and shown to be one of the most potent vasoconstrictors known. Cloning and sequencing of preproendothelin complementary DNA shows that mature endothelin is generated through an unusual proteolytic processing, and regional homologies to a group of neurotoxins suggest that endothelin is an endogenous modulator of voltage-dependent ion channels. Expression of the endothelin gene is regulated by several vasoactive agents, indicating the existence of a novel cardiovascular control system.*

FOLLOWING the discovery in 1980 of endothelium-dependent vasodilation by Furchgott and Zawadzki<sup>1</sup>, vascular endothelium has been recognized as an important functional unit involved in the regulation of vascular smooth muscle tonus. It has been hypothesized that, when stimulated by vasoactive agents such as acetylcholine and bradykinin, endothelial cells (EC) secrete short-lived endothelium-derived relaxing factor(s) (EDRF), causing relaxation of underlying smooth muscle cells (see refs 2 and 3 for reviews). One EDRF has recently been identified as nitric oxide or a closely related substance<sup>4</sup>. Vasoconstriction dependent on or enhanced by intact endothelium has also been observed in response to various chemical and physical stimuli such as, noradrenaline<sup>5</sup>, thrombin<sup>5</sup>, hypoxia<sup>6,7</sup>, increased transmural pressure<sup>8</sup> and mechanical stretch<sup>9</sup>. Augmentation of noradrenaline-induced vasoconstriction by anoxia<sup>5,10</sup> and neuropeptide Y<sup>11</sup> has also been found to be endothelium-dependent. Diffusible factor(s) which mediate these constrictive reactions have yet to be identified. Recent reports have described a protease-sensitive vasoconstrictor activity in supernatants of cultured EC<sup>12-14</sup>. This activity is dependent on the presence of extracellular Ca<sup>2+</sup> and is not affected by blocking the action of  $\alpha$ -adrenergic, cholinergic, serotonergic or histaminergic neurotransmitters. Production of this peptidergic substance by EC is influenced by several of the chemical factors listed above<sup>14,15</sup>.

We have now isolated a potent vasoconstrictor peptide from the culture supernatant of porcine aortic EC, determined its amino-acid sequence, and molecularly cloned the peptide precursor. This peptide, endothelin, does not belong to any previously known peptide family. The endothelin sequence,

however, shows local homologies to a certain group of peptide neurotoxins that act on voltage-dependent Na<sup>+</sup> channels, suggesting that endothelin also acts directly on membrane channels. Studies with preproendothelin cDNA reveal that the precursor peptide is proteolytically processed in an unusual way and that its biosynthesis in cultured EC is regulated at the transcriptional level in response to various chemical and mechanical stimuli.

## Purification and structure

The supernatant from confluent monolayer cultures of porcine aortic EC causes an endothelium-independent, slow-onset contraction when added to porcine coronary artery strips at a final concentration of 10% v/v or more (data not shown). Unconditioned medium, or medium conditioned with human fibroblast IMR-90 cells, has no effect. Pretreatment of the conditioned medium with 1  $\mu$ g ml<sup>-1</sup> trypsin at 37 °C for 2 h abolishes the endothelin activity. The activity is also found in the serum-free conditioned medium, and no significant decrease of the activity was observed after a long-term (4-5 weeks) serum-free maintenance of the EC culture. These observations indicate that endothelin is not a derivative of a serum component.

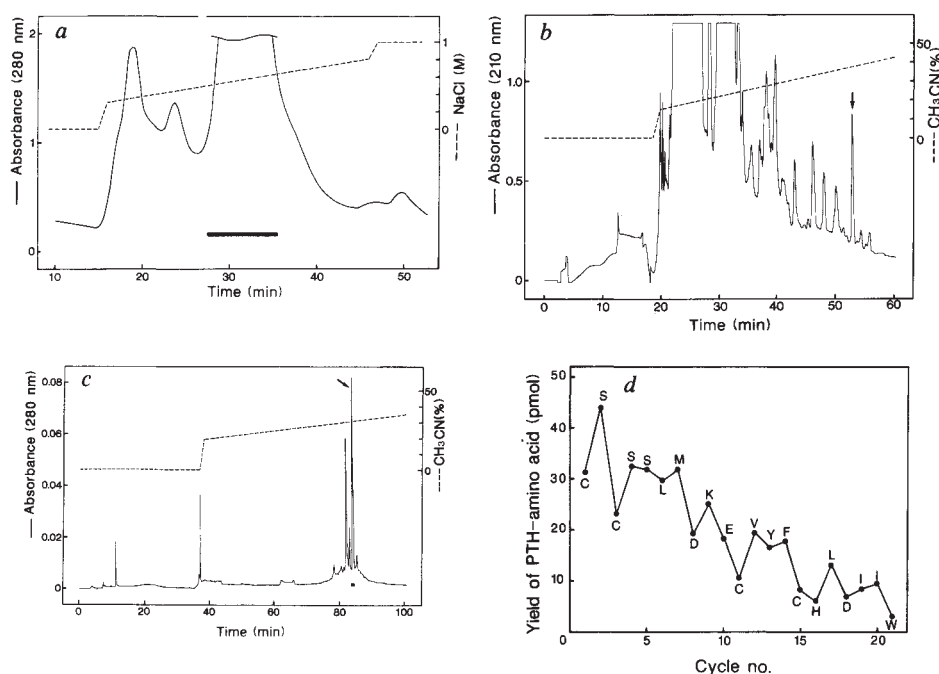
That the confluent EC monolayer can be maintained for several weeks even in protein-free culture medium greatly facilitated its purification. We purified the vasoconstrictor from concentrated serum-free conditioned medium, by collecting active fractions after anion-exchange column chromatography and two steps of reversed-phase HPLC (Fig. 1a-c). The final endothelin fraction, corresponding to the absorbance peak at the arrow in Fig. 1c, was eluted as a single peak on analytical anion-exchange and reversed-phase HPLCs. In one series of experiments, 2.9 nmol purified endothelin was obtained from 9.6 l of the medium conditioned for 5 days with approximately 4  $\times$  10<sup>9</sup> cells. The amino-acid composition, determined by acid hydrolysis, was: Asx, 2.05; Ser, 2.70; Glx, 1.08; Cys, 1.37, (as cystine); Val,

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**Fig. 1** Purification and sequence analysis of porcine endothelin. *a*, Anion-exchange chromatogram of concentrated serum-free EC-conditioned medium. Concentrated medium (20 ml) was directly loaded onto a DEAE-Toyopearlpack 650 M column (2.2 × 20 cm; Tosoh) connected to a Beckman model 342 HPLC system and equilibrated with 20 mM Tris-HCl (pH 7.0), and a linear gradient of NaCl (broken lines) was applied at a flow rate of 4 ml min<sup>-1</sup>. The eluate absorbance at 210 and 280 nm was recorded and the active fraction designated by a solid bar was collected. *b*, Reverse-phase HPLC. The active fraction from the anion-exchange chromatography was directly applied on a Unisil-Q C<sub>18</sub> column (7.6 × 250 mm; Gasukuro-Kogyo) equilibrated with 0.1% trifluoroacetic acid (TFA). A gradient of acetonitrile was used at a flow rate of 3 ml min<sup>-1</sup>. Endothelin activity was co-eluted with the absorbance peak designated with an arrow. *c*, Second reverse-phase HPLC. The active fraction in *b* was further purified on a Chemcosorb 5-ODS-H column (4.6 × 250 mm; Chemco), with a gradient of acetonitrile at a flow rate of 1 ml min<sup>-1</sup> in the presence of 0.1% TFA. Arrow, final endothelin fraction. *d*, Peptide sequence analysis. The yield of phenylthiohydantoin (PTH)-amino acids at each cycle of Edman degradation is shown with the one-letter amino acid notation.



**Methods** Endothelial cells (EC) were isolated from adult porcine thoracic aortas<sup>37</sup> and grown to a confluent monolayer in Eagle's minimum essential medium (MEM), containing 10% horse serum at 37 °C in 5% CO<sub>2</sub>/95% air. Cells were identified by typical 'cobble-stone' morphology and by immunofluorescence to factor VIII antigen. Smooth-muscle cell contamination was less than 0.1%. After passing 10–15 times, cells were grown to confluence in a multi-layered tissue culture flask on a surface area of 12,000 cm<sup>2</sup> (Cellfactory, Nunc). Cells were washed twice with phosphate buffered saline and fed with 3.2 l of serum-free MEM. Medium was changed every 5 days and the conditioned medium was pooled and stored at -20 °C. Pooled conditioned medium was centrifuged at 1,000g for 20 min, the supernatant loaded on a 3 × 18 cm C<sub>18</sub> reverse-phase column (SP-C-ODS, Chemco) equilibrated with 0.1% TFA, and adsorbed material eluted with 30 ml 0.1% TFA/70% acetonitrile. Eluate was extracted twice with 200 ml diethylether and the aqueous phase adjusted to pH 7.0 with Tris base. For bioassay of endothelin activity, dilutions of EC-conditioned medium or the HPLC fractions were added directly into the muscle chamber (see Fig. 2 legend). For peptide sequencing ~100 pmol purified endothelin was reduced in a solution of 0.2 M *N*-methylmorpholine acetate (pH 8.0), 20 mM 2-mercaptoethanol at 22 °C for 30 min. The reaction was incubated at 22 °C for a further 90 min after adding 4-vinylpyridine to 40 mM. The pyridylethylated endothelin was purified on a C<sub>18</sub> reverse-phase HPLC and applied to a gas-phase protein sequencer (Model 470A/120A, Applied Biosystems).

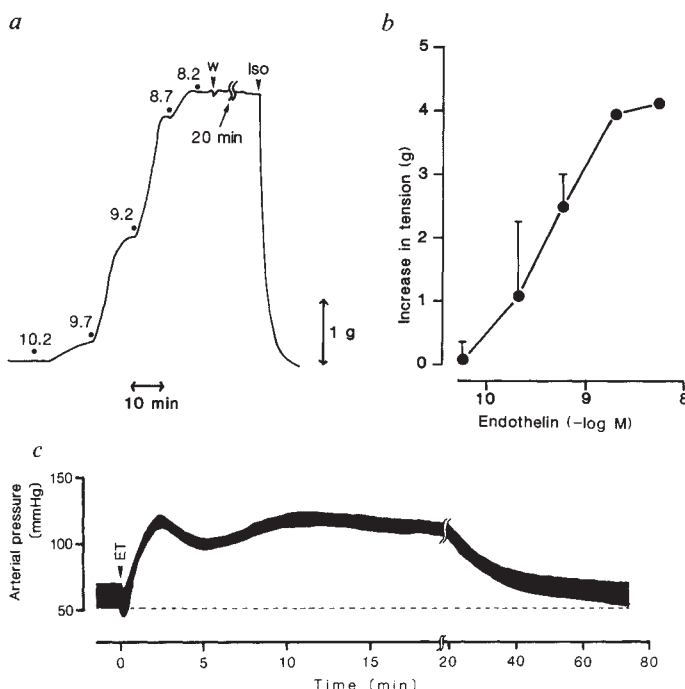
0.91; Met, 0.90; Ile, 1.03; Leu, 1.97; Tyr, 0.64; Phe, 1.11; His, 0.99; Lys, 1.00; Trp, 0.98; Thr, Pro, Gly, Ala and Arg, undetected. Furthermore, automated gas-phase peptide sequencing (Fig. 1d), and carboxyterminal analysis by hydrazinolysis (data not shown) together showed that porcine endothelin is of relative molecular mass (*M<sub>r</sub>*) 2,492, comprised of 21 amino acid residues with free amino- and carboxy-termini. The four cysteine residues of endothelin were found to form two intrachain disulphide bonds (Fig. 5). Synthetic endothelin was prepared by liquid-phase chemistry, crosslinking the four cysteine residues according to the analytically determined structure. The synthetic endothelin showed complete biological activity, and retention times identical to those of the natural peptide on a C<sub>18</sub> reverse-phase HPLC and an anion-exchange HPLC (data not shown). Technical details for the determination of the primary structure and the disulphide-bond topology, together with the liquid-phase synthesis of endothelin will be described elsewhere.

### Vasoconstrictor/pressor activities

A typical example and the dose-response relationship of the vasoconstrictor effect of endothelin on porcine right coronary artery strips is shown in Fig. 2. Similar results were obtained with strips of rat aortas, cat basilar arteries, rabbit mesenteric arteries, dog mesenteric, femoral and renal arteries, and human mesenteric and pulmonary artery branches (data not shown). The maximum tensions are comparable to those of KCl-induced contraction. The estimated concentration at which endothelin

was 50% effective (EC<sub>50</sub>) in this assay was  $4.0 \pm 2.2 \times 10^{-10}$  M. This figure is at least one order of magnitude lower than the reported values for angiotensin II<sup>16</sup>, vasopressin<sup>17</sup> or neuropeptide Y<sup>18</sup>, indicating that endothelin is the most potent mammalian vasoconstrictor peptide known to date. *In vivo*, intravenous bolus injection of endothelin causes a markedly sustained rise in arterial pressure in anaesthetized, chemically denervated rats (Fig. 2c). The pressor effect is more prominent in diastole. Typically, more than 40–60 min is required for return of arterial pressure to the base-line levels.

The endothelin-induced contraction of porcine coronary artery strips is long-lasting and characteristically difficult to wash out, although completely reversed by the addition of  $2 \times 10^{-7}$  M isoproterenol, or  $10^{-6}$  M glyceryl trinitrate (Fig. 2a). The constrictive response is resistant to the following antagonists:  $\alpha$ -adrenergic (phentolamine,  $10^{-6}$  M), H<sub>1</sub>-histaminergic (diphenhydramine,  $10^{-6}$  M), serotonergic (methysergide,  $10^{-6}$  M), cyclooxygenase (indomethacin,  $10^{-5}$  M), and lipooxygenase (nordihydroguaiaretic acid,  $10^{-4}$  M). This suggests that endothelin acts directly on the smooth muscle cells. The endothelin-induced contraction is completely inhibited when bathing solution was substituted with Ca<sup>2+</sup>-free Krebs-Ringer solution containing 1 mM EGTA immediately before the addition of  $10^{-9}$  M endothelin. Furthermore, the vasoconstriction was markedly attenuated in the presence of the Ca<sup>2+</sup>-channel blocker, nifedipine ( $10^{-8}$  M). These findings agree with previous observations<sup>12,13</sup> and suggest that influx of extracellular Ca<sup>2+</sup> is required for the action of endothelin.

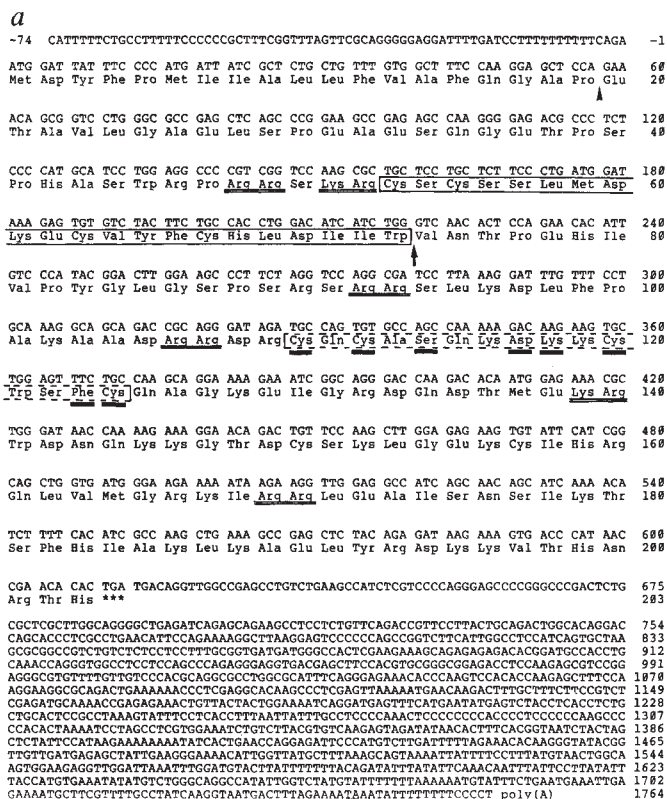


**Fig. 2** *a*, Typical constrictive response of porcine right coronary artery strips to cumulatively applied porcine endothelin. Purified endothelin (quantified by amino-acid analysis) was added to the muscle chamber to give the final concentrations shown (designated in terms of negative log molar). W, wash with Krebs-Ringer solution; Iso,  $2 \times 10^{-7}$  M isoproterenol. *b*, Dose-response relationship of the vasoconstrictor effect ( $n=4$ ). Similar results were obtained with synthetic endothelin. *c*, *In vivo* pressor effect of porcine endothelin in an anaesthetized, chemically denervated rat. One nmol  $\text{kg}^{-1}$  of synthetic endothelin (ET) was applied in bolus intravenously at time 0. Note that the chart speed was changed at 20 min. The base-line diastolic pressure is shown by a broken line. The biphasic response seen in this example was not always observed.

**Methods.** Right proximal coronary arteries were isolated from fresh adult porcine hearts, brought from a local slaughterhouse within 20 min after death in ice-cold Krebs-Ringer solution (113 mM NaCl, 4.8 mM KCl, 2.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 25 mM  $\text{NaHCO}_3$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 5.5 mM glucose). Arterial segments were cut into  $2 \times 15$  mm helical strips with the intima denuded by rubbing with a small swab, and suspended in 3-ml siliconized glass organ chambers filled with Krebs-Ringer solution maintained at  $37^\circ\text{C}$  and gassed with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . Arterial strips were equilibrated at a passive tension of 1.5 g for two hours. The contraction was measured as an increase in isometric tension with force-displacement transducers (model WT-611T, Nihon Koden). The effectiveness of intimal denudation was assessed by demonstrating that the vasodilatory response to  $10^{-9}$  M Substance P was abolished<sup>3</sup>. For the *in vivo* experiment, a 10-week-old male Wistar rat (350 g body weight) was anaesthetized with urethane ( $1 \text{ g kg}^{-1}$  i.p.), and pretreated with atropine ( $0.25 \text{ mg kg}^{-1}$  i.v.), propranolol ( $1 \text{ mg kg}^{-1}$  i.v.) and bunazosin ( $1 \text{ mg kg}^{-1}$  i.v.). The blood pressure was directly recorded from the right carotid artery with a disposable pressure transducer (model SCK-590, Gould).

## Structure of preproendothelin

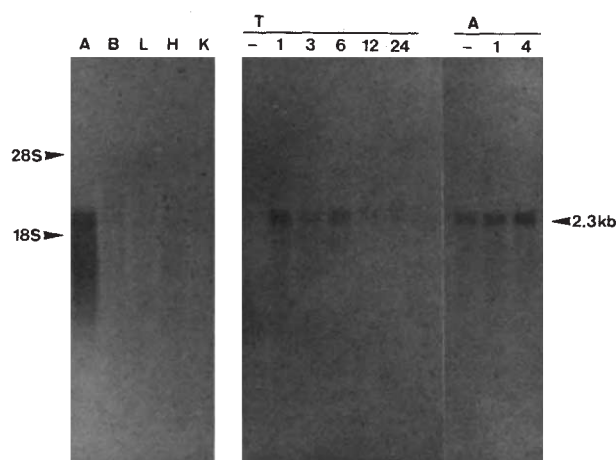
Screening of  $\sim 2 \times 10^6$  clones from a  $\lambda$ gt10 cDNA library constructed for porcine aortic EC mRNA with the synthetic DNA probe, described in Fig. 3 legend, resulted in the identification of 38 hybridization-positive clones. Four of these clones,  $\lambda$ PET2,  $\lambda$ PET4,  $\lambda$ PET5 and  $\lambda$ PET11 were subjected to further characterization. Genomic Southern analysis probed with the 490 base-pair (bp) *Sac*I fragment of  $\lambda$ PET4 insert showed that porcine endothelin is encoded in a single-copy gene (data not shown). The nucleotide sequence of the cDNA inserts is shown in Fig. 3a. The 5'-proximal ATG triplet is followed by the only



**Fig. 3** *a*, Complete nucleotide sequence of porcine preproendothelin cDNA and deduced amino-acid sequence. Arrowhead, putative signal sequence cleavage site predicted by the algorithm described by von Heijne<sup>38</sup>. Paired basic amino-acid residues, Lys-Arg and Arg-Arg, are doubly underlined. Solid lines enclose endothelin sequence; broken lines enclose 'endothelin-like peptide'; solid bars, residues identical to those of endothelin; arrow, the unusual proteolytic processing site at the carboxyl(?)-terminus of endothelin; underline, poly(A) addition signal. *b*, Alignment of endothelin sequence with 'endothelin-like peptide' (ELP) and the amino-terminal regions of several  $\alpha$ -scorpion toxins<sup>30</sup>. One-letter amino acid notation. Half-cysteine residues are shown in bold; boxes, identities or conserved amino-acid substitutions<sup>39</sup>. Be M10, *Buthus epeus* neurotoxin M10; BoT III, *Buthus occitanus tunetanus* neurotoxin III; Lq IV and V, *Leiurus quinquestriatus quinquestriatus* neurotoxin IV and V; AaH III, *Androctonus australis Hector* neurotoxin III.

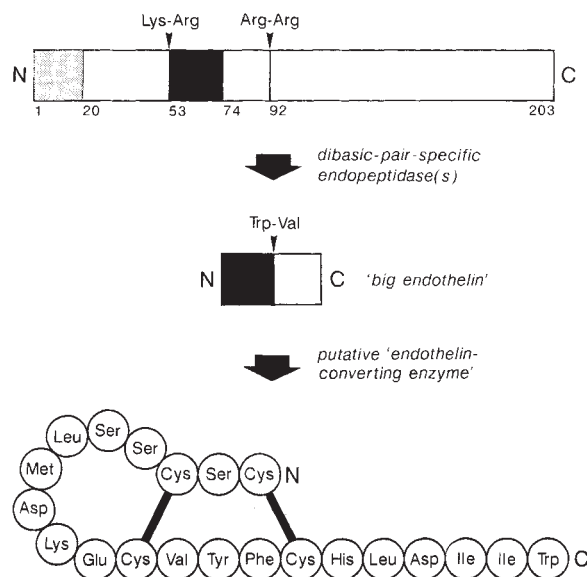
**Methods.** RNA was extracted from porcine aortic EC and poly(A) RNA was selected by oligo(dT) cellulose column chromatography<sup>40</sup>. A cDNA library of  $2 \times 10^6$  independent clones was constructed<sup>41</sup> in  $\lambda$ gt10 from 2  $\mu\text{g}$  of the poly(A) RNA. The unamplified library was screened<sup>40</sup> with a single 'optimal' synthetic probe encoding amino-acid residues 7-20 of endothelin. Oligonucleotides ATGGACAAGGAGTGTGTCTACTTCTG and GATGATGTCCAGATGGCAGAGTAGA were synthesized according to the mammalian codon usage statistics<sup>42</sup> with Applied Biosystems model 380A DNA synthesizer, annealed to each other, and labelled<sup>43</sup> to a specific activity of  $10^9$  c.p.m.  $\mu\text{g}^{-1}$  with Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of [ $\alpha$ - $^{32}\text{P}$ ]dCTP (3,000 Ci  $\text{mmol}^{-1}$ , Amersham). Hybridization was carried out at  $42^\circ\text{C}$  in 20% formamide. Membranes were washed in  $0.2 \times \text{SSC}$  at  $30^\circ\text{C}$ . The restriction fragments of cDNA inserts of four of the hybridization-positive phages,  $\lambda$ PET2,  $\lambda$ PET4,  $\lambda$ PET5 and  $\lambda$ PET11, were subcloned into plasmid pUC118 or pUC119. The extent of the four cDNA inserts were:  $\lambda$ PET2, nucleotide -74 to 501;  $\lambda$ PET4, -2 to poly(A);  $\lambda$ PET5, 62 to poly(A);  $\lambda$ PET11, -13 to poly(A). The recombinant plasmids were rescued as single-stranded DNA and sequenced by the dideoxy-chain-termination method<sup>44</sup>. The complete sequences of the four inserts were independently determined, both strands being completely covered in the translated regions.





**Fig. 4** Northern analysis of preproendothelin mRNA from various porcine tissues (left) and cultured porcine aortic EC exposed to thrombin or the  $\text{Ca}^{2+}$  ionophore A23187 (right). Tissue RNA (20  $\mu\text{g}$  per lane) was extracted by the LiCl-urea method<sup>45</sup> from adult porcine aortic intima (A), whole brain (B), lung (L), right atrium (H), and kidney (K). As many macroscopic blood vessels as possible were removed from the tissues.

Intimal tissue was scraped off from fresh porcine thoracic aortas directly into the LiCl-Urea lysis solution. RNA was electrophoresed through a 1.2% agarose/formaldehyde gels, transferred to GeneScreen Plus membranes (NEN) and hybridized as recommended by the manufacturer with the insert of  $\lambda\text{PET4}$  labeled using [ $\alpha\text{-}^{32}\text{P}$ ]dCTP (3,000 Ci  $\text{mmol}^{-1}$ , Amersham) to a specific activity of  $8 \times 10^8$  c.p.m.  $\mu\text{g}^{-1}$  by the random-primed labelling method<sup>46</sup>. The autoradiograph was exposed for 7 days. RNA from confluent EC (5  $\mu\text{g}$  per lane) was extracted immediately before (-), and at the designated number of hours after, the addition of 2 NIH U  $\text{ml}^{-1}$  bovine thrombin (T) or  $10^{-6}\text{M}$  A23187 (A). Autoradiographs were exposed for 10 h. After autoradiography, the membranes were rehybridized with a myosin regulatory light-chain cDNA pGMRL7E1 (our unpublished work) for an internal standard of total mRNA content; all lanes in each series of experiments showed similar signal intensities. Signal intensities cannot be directly compared between the thrombin series and the A23187 series as different stocks of EC were used.



**Fig. 5** Possible pathway of endothelin biosynthesis. The putative secretory signal sequence and the endothelin sequence are shown by the shaded and closed boxes, respectively. The rest of the preendothelin sequence is indicated by an open box. A 39-amino-acid residue intermediate, or 'big endothelin', is considered to be generated from preendothelin by the proteolytic cleavages at paired basic residues. Mature endothelin is then produced through an unusual, previously unknown processing by a putative 'endothelin converting enzyme'.

A presumptive pathway of the production of mature endothelin is schematically presented in Fig. 5.

Interestingly, the sequence of preproendothelin from Cys 110 to Cys 124 shows a significant homology to the amino-terminal 15 residues of endothelin (Fig. 3b). Eight out of 15 residues (53%) are identical and, more significantly, the relative positions of the four cysteine residues are perfectly conserved. Furthermore, this sequence is flanked with the dibasic-pairs Arg 106-Arg 107 and Lys 139-Arg 140. It is possible that this 'endothelin-like peptide' is actually secreted from the EC, and that it possesses some biological activity that was undetectable in the vasoconstriction bioassay used in the purification procedure. Just prior to the stop codon is a potential glycosylation site (Asn-X-Thr/Ser) at residue 200.

## Expression of preproendothelin mRNA

Northern blots probed with  $\lambda\text{PET4}$  insert showed that a single 2.3 kilobase (kb) preproendothelin mRNA was expressed not only in the cultured EC but also in porcine aortic intima *in situ* (Fig. 4). Preproendothelin mRNA was not detected in porcine brain, atrium, lung or kidney. This implies that little endothelin is produced by the EC of the microvasculature within these tissues and is consistent with the observation that medium conditioned by microvascular EC from human omental fat does not cause significant vasoconstriction<sup>14</sup>.

To establish whether the production of endothelin is constitutive or inducible, confluent monolayers of porcine aortic EC were exposed for specified time periods to medium containing various vasoactive agents. Total cellular RNA was extracted from the EC and the amount of preproendothelin mRNA monitored by Northern analysis (Fig. 4). The level of preproendothelin mRNA increased significantly within 1 h of adding thrombin (2 U  $\text{ml}^{-1}$ ) or the  $\text{Ca}^{2+}$ -ionophore A23187 ( $10^{-6}\text{M}$ ). Adrenaline ( $10^{-6}\text{M}$ ) also caused a marked induction of preproendothelin mRNA within 1 h (H.K., *et al.*, manuscript in preparation). These agents are known to cause endothelium-dependent

long open-reading frame, which includes the endothelin sequence. The sequence around this ATG fits the consensus sequence for eukaryotic translation initiation sites<sup>19</sup> and the first 19 residues of the deduced amino-acid sequence are characteristic of a secretory signal sequence, that is a hydrophobic core followed by residues with small polar side-chains<sup>20</sup>. The 203-residue preproendothelin sequence deduced from this long open-reading frame is shown under the nucleotide sequence in Fig. 3a. The existence in vascular endothelium of mRNA encoding the prepro- form of endothelin indicates that endothelin is produced by *de novo* synthesis and processing in a manner similar to that of many peptide hormones and neuropeptides.

As anticipated, paired basic amino-acid residues Lys 51-Arg 52, which are recognized by processing endopeptidases<sup>21</sup>, directly precede the endothelin sequence (Fig. 3a). Surprisingly, however, no dibasic-pair is found thereafter until Arg 92-Arg 93. This indicates that the mature endothelin found in the EC-conditioned medium is generated via previously unknown, unusual proteolytic processing between Trp 73 and Val 74 presumably involving an endopeptidase with a chymotrypsin-like specificity. Artefactual proteolysis during the peptide purification is unlikely because the vasoconstrictor activity is always exclusively associated with 21-residue endothelin (Fig. 1; 4 separate experiments). This putative 'endothelin-converting enzyme', could be present in one of three forms: (1) a soluble intracellular enzyme; (2) membrane-bound like the angiotensin converting enzyme<sup>22</sup>; or (3), secreted and active outside the cell.

vasodilation because of the production of EDRF<sup>3</sup> and, in some cases, to cause endothelium-dependent constriction<sup>2,15</sup>. These latter findings are here substantiated by the observed induction of the endothelium-derived constrictor molecule. In addition, preliminary experiments showed that when EC were cultured in the presence of a medium flow and thus placed under a chronic fluid-mechanical 'shear stress', expression of the prepro-endothelin gene is significantly down-regulated (M. Yoshizumi, personal communication). The flow-dependent inhibition of endothelin production could contribute to endothelium-mediated, flow-induced vasodilatory responses<sup>23,24</sup>.

Endothelial cells have few secretory granules when examined under the electron microscope<sup>25</sup>. O'Brien *et al.*<sup>14</sup> detected little vasoconstrictor activity in freeze-thaw lysates of the EC that had released the peptidergic constrictor activity into the medium. It is therefore unlikely that endothelin is accumulated in granules and released in response to stimuli. The production of endothelin seems to be regulated predominantly at the level of mRNA transcription.

## Physiological implications

The endothelin-type of structure with multiple disulphide bonds within a single, relatively short peptide chain is previously unknown among bioactive peptides of mammalian origin. This type of configuration is often found in a certain group of peptide toxins that act on membrane channels, such as apamin from bee venoms<sup>26</sup>, conotoxins from the venoms of fish-hunting sea snails<sup>27</sup> and neurotoxins of scorpion venoms<sup>28</sup>. An examination of the National Biomedical Research Foundation database with the algorithm of Lipman and Pearson<sup>29</sup> detected significant regional homologies between endothelin and  $\alpha$ -scorpion toxins<sup>30,31</sup> (Fig. 3b). The  $\alpha$ -scorpion toxins are ~7,500 M<sub>r</sub> peptides with four intrachain disulphide bridges. They bind to tetrodotoxin-sensitive Na<sup>+</sup> channels and inhibit the inactivation of the activated channels, thereby blocking neuronal transmission<sup>32</sup>. These findings raise the possibility that endothelin, like these neurotoxins, acts directly on membrane ion channels. The absolute dependency of endothelin-induced vasoconstriction

on the presence of extracellular Ca<sup>2+</sup>, in conjunction with the inhibition of the endothelin-induced contraction by low doses of nicardipine, suggests that the endothelin action is closely associated with the Ca<sup>2+</sup> influx through the dihydropyridine-sensitive Ca<sup>2+</sup> channels. The dihydropyridine-sensitive Ca<sup>2+</sup> channels and the tetrodotoxin-sensitive Na<sup>+</sup> channels have recently been found to belong to the same superfamily of voltage-dependent membrane ion channels<sup>33</sup>. Although further studies are required for the elucidation of the vasoconstriction mechanism, it is tempting to hypothesize that endothelin is an endogenous agonist of the dihydropyridine-sensitive Ca<sup>2+</sup> channels.

The observations presented in this study suggest that a novel endothelium-mediated regulation exists in the mammalian cardiovascular system. The basal tonus of vascular beds *in vivo* can be influenced by the level of endothelin production, because endothelin mRNA is actively expressed in EC *in situ* (Fig. 4). Our results support the previous findings that EC produces both EDRF and endothelin in response to various chemical and haemodynamical conditions<sup>34,35</sup>. However, the time-course of the induction and the decay of these mutually antagonizing substances could differ considerably. EDRF may be involved in the rapid local control of vascular tonus, whereas endothelin may contribute to a long-term regulation. The nature of *in vivo* endothelin production requires further study, as cultured EC may approximate more closely to a state of 'injury' *in vivo*<sup>36</sup>. Endothelin, having a potent, strong and characteristically long-lasting vasoconstrictor activity, may be important in the control of systemic blood pressure and/or local blood flow: disturbances in the control of endothelin production could contribute to the pathogenesis of hypertension and that of pathological vascular spasm.

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## The spin histories of PSR1821–24 in M28 and PSR1951+32 in CTB80

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A popular interpretation of the period versus period derivative distribution of the majority of radio pulsars is that the underlying neutron stars are born rapidly spinning, with surface magnetic fields of  $10^{12}$ – $10^{13}$  G that decay exponentially on a timescale of  $10^6$ – $10^7$  yr (ref. 1). A new class of radio pulsing neutron stars with rapid rotation periods,  $<11$  ms, but low magnetic fields,  $10^8$ – $10^{10}$  G, may have been spun-up (recycled) by the accretion of material from a binary companion<sup>2–7</sup>. Compelling support for this comes from the fact that in several cases the pulsar is in a binary system with a degenerate companion, the burnt-out remains of the mass donor<sup>6–8</sup>. In those cases where the pulsar is single, it seems likely that the binary orbit has been disrupted. The rotation period of the recently discovered 3.05-ms pulsar PSR1821–24 in the globular cluster M28 (ref. 9) appears to violate the minimum possible from spin-up by accretion, for the inferred magnetic field strength of  $2.3 \times 10^9$  G (ref. 10). In addition, the inferred magnetic field strength of  $5 \times 10^{11}$  G of PSR1951+32, in the supernova remnant CTB80 (ref. 11), is low when compared to the spin-down age of  $\sim 10^5$  yr (ref. 12), suggesting perhaps that not all neutron stars are born with magnetic fields  $>10^{12}$  G. We point out here that these apparent contradictions arise because the derivation of the previously used spin-up limit does not take into account the domination of the inner accretion disk by radiation pressure. The correct relation depends on the underlying assumptions about the viscosity in the inner disk and how the accretion disk interacts with the magnetosphere.

An accreting, magnetized neutron star will be spun-up by the angular momentum of the accreted material until its rotation period equals the last keplerian period of the accretion disk at the magnetosphere boundary. If the neutron star rotates faster than this equilibrium period, the centrifugal drag exerted by the magnetosphere prevents accretion onto the neutron star surface. In the past the radius of the magnetospheric boundary has been estimated by equating the free-fall ram pressure of the accretion flow to the pressure of the neutron star dipole magnetic field<sup>3</sup>. A spherical accretion flow is assumed. Ghosh and Lamb<sup>13</sup>, hereafter GL, have shown that a very similar expression holds for the more relevant case where an accretion disk mediates the flow, and that the relation for a spherical accretion can be used as a good approximation. We have recently shown in ref. 14, hereafter paper I, that this is only true for the outer gas pressure-dominated regions of an accretion disk. In the inner regions of a standard  $\alpha$ -viscosity accretion disk, radiation pressure determines the structure<sup>15</sup>, and the relation for the magnetospheric radius derived for the spherical case cannot be used. When  $\dot{M}_e > 8 \times 10^{-2} \alpha^{-1/11} \dot{M}_x^{-16/11} \mu_{28}^{5/11}$  the disk region outside the magnetosphere will be dominated by radiation pressure. (The mass accretion rate,  $\dot{M}_e$ , is expressed in terms of the Eddington limit, with a 6% mass to energy conversion efficiency, onto an object with a mass  $M_x$  in solar masses and a magnetic dipole moment of  $\mu_{28}$ , in units of  $10^{28}$  G cm<sup>3</sup>). The parameter  $\alpha$  is the scaling factor between the viscous stress and the disk pressure and is  $\leq 1$  (ref. 15). As pointed out in paper I, this regime is relevant to the recently discovered fast pulsars with  $\mu_{28} \leq 100$  ( $B \leq 10^{12}$  G for a neutron star of 10 km radius).

The structure of the inner radiation pressure-dominated disk and its interaction with a magnetosphere are very uncertain (see paper I). One uncertainty is because accretion disk models assume that the viscous stress, responsible for transporting angular momentum outward through the disk, is proportional to the pressure in the disk<sup>15</sup>. In the inner radiation pressure-dominated region it is debatable whether this scaling should refer to the radiation plus gas pressure, or only to the gas pressure<sup>16</sup>. Because the radiation pressure can be several orders of magnitude greater than the gas pressure this can make a great difference. If the viscous stress scales with the radiation pressure (case I) then the disk solution will be unstable to secular and thermal instabilities<sup>17</sup>. If the gas pressure alone determines the viscosity (case II), then the disk is stable, but the internal structure and emerging spectrum of the radiation pressure-dominated region will be different from that given by Shakura and Sunyaev<sup>18</sup>. Another problem is how the disk interacts with the magnetosphere. This is a controversial topic which centres on whether or not the stellar magnetic field is completely screened from the disk<sup>19</sup>. It is unclear whether pressure equilibrium or some other criterion, such as the flow of angular momentum from the disk to the magnetosphere, determines the magnetospheric radius (see paper I for a more complete discussion).

Expressions for the magnetospheric radius and equilibrium period were given in paper I for cases I and II assuming that the magnetospheric radius occurs where the internal disk pressure equals the magnetic pressure (assuming a perfectly diamagnetic disk and an unperturbed dipole field). Very different results are obtained for the two cases. For case I (radiation pressure scaling), the magnetospheric radius is independent of mass accretion rate. In case II (gas pressure scaling), the dependence of the radius on mass accretion rate is  $\dot{M}^{-2/3}$ , with the radius 10 times smaller than the value given by the equation derived for the gas pressure-dominated region of the disk with the same accretion rate and magnetic field strength. In paper I we obtained the following expressions for the equilibrium period,  $P_{eq}$ . In case I

$$P_{eq}(I) = 8.2 \times 10^{-3} \alpha^{1/3} \dot{M}_x^{4/3} \mu_{28}^{2/3} \text{ s.} \quad (1)$$

(note that there is no dependence on  $\dot{M}_e$ ). In case II

$$P_{eq}(II) = 0.12 \times 10^{-3} \alpha^{1/2} \dot{M}_x^{3/2} \dot{M}_e^{-1} Y^{-1} \mu_{28}^{5/4} \text{ s.} \quad (2)$$

The parameter  $Y = (1 - \xi)$ , where  $\xi$  is the fraction of the keplerian angular momentum at the magnetosphere boundary that is captured by the neutron star<sup>20</sup>, and can be assumed to be  $\sim 1$ . In a different approach to the magnetosphere/accretion disk interaction, GL assumed that partial threading of the disk by the stellar magnetic field occurs in an extended boundary layer, wherein the disk angular momentum is transferred to the neutron star. This solution is primarily dependent on the variation in the disk thickness with radius, which is the same for both cases (paper I). This gives

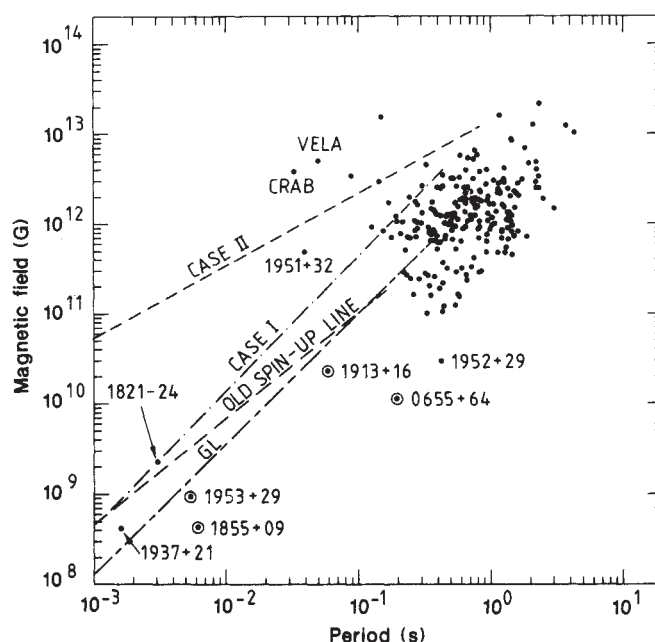
$$P_{eq}(GL) = 17.9 \times 10^{-3} \dot{M}_x^{-4/5} \dot{M}_e^{-3/25} \mu_{28}^{18/25} f(\omega_{crit})^{3/2} Y^{6/25} \text{ s.} \quad (3)$$

where  $f(\omega_{crit}) = (1 - \omega_s)^{-4/25} (1 + \omega_s)^{-2/25}$  and  $\omega_s$  is a fastness parameter defined as the ratio of the stellar and keplerian angular velocities at the magnetospheric boundary<sup>13</sup>. Close to the equilibrium period,  $f(\omega_{crit}) \approx 1.2$  (F. K. Lamb, personal communication in paper I). Note that equation (3) is independent of  $\alpha$ .

In Fig. 1 we plot as a function of spin period the inferred magnetic field strength for all the radio pulsars given by Manchester and Taylor<sup>21</sup>, as well as the recently discovered binary and single millisecond pulsars<sup>6,9,11</sup>. Also shown are the equilibrium periods given by equations (1), (2) and (3), as well as the previously used equilibrium period that assumes the

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**Fig. 1** The magnetic field inferred from the period and period derivatives for the sample of radio pulsars given in ref. 21, plotted as a function of rotation period. Also shown are the recently discovered single and binary millisecond pulsars<sup>6,9,11</sup>. (For clarity we have not shown the binary pulsars PSR2303+46 and PSR0820+02, which have periods of 1.1 s and 0.864 s and magnetic field strengths of  $6 \times 10^{11}$  G and  $3 \times 10^{11}$  G respectively.) The various spin-up limits, defined by equations 1, 2 and 3 are also shown. The parameters used were  $M_x = 1$ ,  $\alpha = 1$  and  $M_e = 1$ . A neutron star radius of 10 km was used to convert from  $\mu_{28}$ . Also shown is the 'old spin-up limit' which uses the magnetospheric radius given for spherical infall (ref. 3) with  $M_x = 1.4 M_e$  and  $M_e = 1$ .



magnetospheric radius from the spherical case ('old spin-up limit'). For each accretion disk/magnetospheric model these relations provide a limit and, if the model is correct, no recycled radio pulsar should lie to the left of the limit. Where appropriate we assume  $\alpha = 1$  and  $M_e = 1$ . Decreasing  $\alpha$  has no effect on the GL limit, but will move the case I and II lines to the left, increasing each allowed region. This is a consequence of the fact that if the viscosity is decreased, an increased disk pressure is required to support a given accretion rate. Decreasing  $M_e$  will move the case II and GL lines to the right, decreasing each allowed region, but has no effect on case I. Variations in the mass and radius of the neutron star can also change the value of the equilibrium period, but the magnitude of this uncertainty is small when compared to the other effects.

The observed 3.05-ms period of PSR1821-24 violates the previously used spin-up limit which, for the inferred magnetic field strength<sup>10</sup>, is  $\sim 3.8$  ms for a  $1.4 M_\odot$  star accreting at the Eddington limit (Fig. 1). The gradient of the line defining the minimum spin-up period for case I, shown in Fig. 1, is somewhat steeper than the old spin-up limit, but its overall location is similar and PSR1821-24 lies on the limit for  $\alpha \approx 1$ . The GL model is not consistent with the position in Fig. 1 of PSR1821-24. (It also excludes the 1.5-ms pulsar PSR1937+21, although this single pulsar might have been formed by the coalescence of two neutron stars; see ref. 7 and references therein.) The relation for case II gives a much larger allowed region in Fig. 1 with PSR1821-24 well to the right of the line in the allowed region. Figure 1 illustrates how the recycled radio pulsars can be used to gain insight into the nature of the viscosity in the inner regions of accretion disks. For both case I and II an additional degree of freedom is the unknown quantity  $\alpha$ , which, if it is less than unity, will move the spin-up limit to the left and increase the allowed region in Fig. 1. The period and period derivative of PSR1821-24 are consistent with it having been spun-up during a previous interval of accretion for both case I and case II, but not for the GL model for the two accretion disk models applied here.

The spin-down age of PSR1821-24,  $\sim 3 \times 10^7$  yr, is remarkably young for a single millisecond neutron star located in a globular cluster, because it requires that both the end of the mass transfer interval and the disruption of the binary occurred relatively recently when compared to the age of the cluster. Verbunt *et al.*<sup>22</sup> point out that a third star interaction and subsequent expulsion of the neutron star should each occur only

once in the lifetime of the cluster. It seems more likely that the binary disruption occurred during the spin-up phase, or even that the spin-up resulted from the coalescence of a white dwarf and a neutron star, caused by a neutron star that collided with a star towards the end of the giant branch<sup>23,24</sup>, or the direct collision of a neutron star with a main sequence dwarf (see ref. 23 for a full discussion of the various possibilities). In both cases the spin-up would have occurred as the remains of the normal star was accreted onto the neutron star.

Because the allowed region for recycled pulsars can now be much larger, it allows the possibility that the recently discovered 39.5-ms pulsar PSR1951+32 in the supernova remnant CTB80 is also a recycled pulsar. This model would be fully consistent with a case II disk for any value of  $\alpha \leq 1$ , but for case I only if  $\alpha < 3 \times 10^{-2}$  (from equation 1). This pulsar has a spin-down age of  $< 10^5$  yr, and an inferred surface magnetic field strength of  $\sim 5 \times 10^{11}$  G (refs. 11, 12). This is not consistent with the idea that all neutron stars are born with field strengths of a few  $10^{12}$  G, which then decay on a timescale of a few times  $10^6$  yr. An interesting point is that PSR1951+32 has a high velocity,  $\sim 300$  km s<sup>-1</sup> (ref. 12), suggesting either an asymmetric supernova explosion, or that PSR1951+32 was originally part of a binary system that was disrupted by a supernova. The possibility that PSR1951+32 may have been recycled in a binary system is an alternative explanation for its low magnetic field compared to the apparently young spin-down age (as opposed to the possibility it was simply born that way). The magnetic field of PSR1951+32 would have decayed during the previous evolutionary phase when it would have been an X-ray binary pulsar (similar, perhaps, to the 69-ms transient X-ray pulsar A0538-66 in the LMC<sup>25-27</sup>). The time required for the magnetic field to decay by a factor of 10 is  $\sim 10^7$  yr, which is consistent with the lifetime of an X-ray binary containing a Be star<sup>28</sup>. The morphology of the CTB80 remnant in which PSR1951+32 is located is unusual, and comprises an east-west ridge  $\sim 1^\circ$  in extent, with the pulsar embedded at the western end, and to the north and southwest of the pulsar two extensions each  $\sim 0.5^\circ$  in extent<sup>29,30</sup>. Assuming a favourable beaming orientation it would be worthwhile searching for the remains of a previous binary companion to PSR1951+32—another pulsar—elsewhere in its vicinity (PSR1951+32 can have moved a maximum of  $\sim 1^\circ$  in  $10^5$  yr), although the luminosity of a second pulsar born with a surface magnetic field of  $10^{12}$ – $10^{13}$  G could, after  $10^5$  yr, be as much as 10–100 times lower than that of PSR1951+32<sup>21</sup>.



In conclusion, the previously used equation for the minimum possible period for the spin-up by accretion of an accreting magnetized neutron star is not correct and should not be considered a 'hard' barrier when considering the magnetic field-period distribution of recycled radio pulsars. The rapid rotation period and period derivative of both PSR1821-24 in M28 and perhaps also PSR1951+32 in CTB80 are consistent with both having undergone a previous interval of accretion. These pulsars, combined with future discoveries of recycled radio pulsars, can be used to set important limits on viscosity in the radiation dominated region of accretion disks and the nature of the disk-magnetosphere interaction.

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## A synchrotron model for the X-ray emission from supernova 1987A

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An X-ray source coincident with supernova 1987A has recently been discovered by Ginga<sup>1,2</sup> and by the Roentgen Observatory on board the space station Mir-Kvant<sup>3</sup>. To model the X-ray emission, including the temporary variations reported<sup>4</sup>, we propose that a synchrotron nebula has been formed by a pulsar and that the supernova envelope is fragmented. This would permit the central region to be seen directly, leading to a secular light curve on which transient obscurations are superimposed.

The X-ray emission from the supernova was undetected for several months following the optical outburst, but started to brighten at the end of June 1987. Despite some remaining uncertainties in the data, the photon spectrum above 20 keV appears unusually hard and extends up to at least 300 keV. More precisely, the distribution of photons is extremely flat between 10 and 20 keV, whereas at higher energies the photon spectral index is close to 1.4.

The situation is more controversial at lower energies, below 10 keV. Here, the data from Ginga show a spectrum compatible with bremsstrahlung at  $T \approx 4$  keV, or with a power law of index  $\sim 1.8$ . But some upper limits from Roentgen at low energy are barely compatible with these results. If confirmed, these discrepancies could, in principle, be eliminated if the source changes its intensity in the appropriate energy range. Some evidence for short-term variability does indeed exist: measurements made three weeks apart last September show a marked drop in the low energy range, followed by the resumption of the original level in October<sup>4</sup>. A simultaneous decrease in the 12-20-keV flux is of marginal significance.

Another similar event was discovered subsequently in the same energy range (Y. Tanaka, personal communication), and it is possible that a dip also occurred in the 20-40-keV band at the end of October (R. Sunyaev, personal communication).

Several models for the X-ray emission are possible. The first, which was proposed even before the detection of X-rays<sup>5-7</sup>, is based on the Compton diffusion of  $\gamma$ -ray photons, emitted by either radioactive cobalt or a central pulsar. On their path outwards, photons lose energy when scattered by electrons. They will escape only if they can reach the outer regions of the envelope before their energy falls too much, otherwise they will be absorbed by photoelectric opacity.

The main advantage of this model, in its original version, is that *ad hoc* assumptions are not required, and free parameters need not be tuned, because the power of the central source is determined from the optical light curve, which is assumed to be energized by the  $\gamma$ -ray source.

The most serious failure, on the other hand, occurs below 10 keV, where the model predicts a low-energy cut-off, whereas the Ginga results show a rise of the spectrum. The additional flux has been attributed to a different component of the source, namely circumstellar gas heated up to 4 keV by the supernova shock<sup>4</sup>. In our opinion, this interpretation meets some difficulties. In particular, the near coincidence of the rise epoch for the low-energy emission below 10 keV (resulting from hot gas) and above 10 keV (from comptonization of  $\gamma$ -rays) is puzzling, and there is no evidence of the strong radio emission expected when the shock reaches the circumstellar material.

The variability is also a severe problem for the diffusion model. The occurrence of 'dips' strongly suggests that one is observing eclipses caused by intervening material; this is consistent with the finding that at least in one occurrence the variations are less pronounced (or even non-existent) at higher energies. If the obscuring material is moving at speed  $v < v_{\text{exp}}$  ( $v_{\text{exp}} \approx 5 \times 10^8$  cm s<sup>-1</sup> is the remnant expansion velocity), and if  $\Delta t$  is the timescale of the variations, the size of the X-ray source should be  $< v_{\text{exp}} \Delta t \approx 10^{15}$  cm, much smaller than the size of the remnant ( $10^{16}$  cm). The idea that the X-rays arise from reprocessing throughout the entire envelope then runs into serious difficulties.

Further difficulties arise from the observed light curve. The X-ray flux has become detectable sooner and has reached lower values than expected, and has not so far shown any hint of decline. Part of this can be cured by changing the input parameters with respect to the original proposal. In particular, it can be assumed that the optical depths seen by the various parcels of radioactive material are lower, and are distributed over a range of values<sup>8,9</sup>. Then the onset of X-ray emission occurs earlier and the top of the light curve becomes more rounded. By the same token, however, the X-rays should by now be declining, whereas the most recent observations indicate approximate constancy (R. Sunyaev, Y. Tanaka and J. Trumper, personal communications). Furthermore, at earlier times than in the original estimates,  $\gamma$ -ray lines should emerge, and should reach very conspicuous fluxes, much larger than actually measured<sup>10</sup>.

We propose that an alternative source of X-ray emission could be provided by a rotating neutron star. An active pulsar would

produce a large-scale magnetic field and a flow of relativistic particles, giving rise to an extended region of synchrotron emission. Below, we investigate a model where the X-rays result from the direct, unscattered radiation from such a synchrotron nebula, assuming that the remnant is already fragmented into filaments.

The properties of a 'pulsar bubble' or plerion have been investigated in the past by computing the evolution from the early phases until the formation of a fully developed Crab-like supernova remnant<sup>11,12</sup>. Very strong synchrotron emission is expected, ranging from radio frequencies up to high-energy photons. Previously we have suggested that the radio emission detected from type II supernovae several months after the explosion could result from the plerion<sup>12,13</sup>; here we investigate the properties of the X-ray emission. We base our discussion on a simplified model where the bubble of relativistic fluid extends up to the shell and expands with constant velocity. Although more elaborate dynamic models have been considered<sup>14</sup>, we think that the most simple model should be used to guide comparison between observations and theory. We also assume that the pulsar spin-down time is longer than the present age of the remnant, and that the injected electron spectrum obeys a simple power law, with spectral index  $\gamma$  up to a maximum energy  $E_{\max} \propto B_0 P^{-2}$ .

For any reasonable choice of the parameters, the electrons emitting at X-ray energies are dominated by the radioactive losses, so  $S_\nu \propto t^{\beta-\alpha}$ ;  $\beta = 0.5$  for  $\gamma \leq 1$ ,  $\beta = 1 - \gamma/2$  for  $\gamma > 1$ ;  $\alpha = 0.5$  for  $\gamma \leq 1$ ,  $\alpha = \gamma/2$  for  $\gamma > 1$ .

Note that  $\alpha \geq 0.5$  unless continuous acceleration is invoked; this is not incompatible with the spectral shape observed in hard X-rays. Also, the rate of increase predicted for  $\gamma \leq 1$  (~8% per month six months after the explosion, 4% per month one year after) is consistent with the observed secular behaviour.

The main objection that has been raised in the past to this sort of model has concerned the importance of opacity effects, which could completely hide the central nebula for many years or even decades, unless the envelope is severely fragmented. In our opinion the early formation of filaments or individual clouds is a possibility which deserves consideration. The physical agency is possibly Rayleigh-Taylor instability at the interface between the relativistic fluid and the ejecta; the dynamical models alluded to previously<sup>14</sup> predict that the interface is accelerated, so that an effective gravitational field is established with the right sense.

Once early fragmentation of the remnant is given, qualitative solutions to the previous difficulties are easily found. The emergence of low-energy X-rays is not a problem if the residual material between the shell fragments is sufficiently thin. The onset time, broadly correlated with the hard X-rays, is explained, as both components become visible because of the same fragmentation process. The timescale and the nature of the variations are understandable, if one invokes occultation of the synchrotron nebula by filaments having non-radial velocity components. Note that the radiative lifetime of X-ray-emitting electrons turns out to be much shorter than the light travel time across the remnant (see below), so, if the injection is directly from the pulsar magnetosphere, and is not spatially distributed, the X-ray nebula is much smaller than the remnant itself.

The spectral behaviour of the source during an occultation gives information about the extension and thickness of the filaments. If all photons below a threshold energy were cut off, for instance, with different thresholds for different events, this would imply the existence of filaments with different total opacities. If, instead, at all energies the flux were reduced by comparable factors, all the clouds would have to be of a size comparable to the source, and an infinite opacity, and the reduction factor would be determined simply by the geometrical shadow. All kinds of intermediate cases can be imagined, especially if the source dimensions are of a function of

frequency. Finally, a continuing pulsar input is the most natural explanation for the persistence of the X-ray emission.

For the sake of completeness, we have computed numerically the spectrum radiated by a nebula fed by a central pulsar with the same magnetic field as the Crab,  $B_0 = 4 \times 10^{12}$  G. The calculations refer to a nebula expanding with  $v = 5 \times 10^8$  cm s<sup>-1</sup>, and the injection index  $\gamma$  is taken equal to 1. The only adjustable parameter is the period  $P_0$ ; we have chosen  $P_0 = 18$  ms, to fit the measured flux from 40 keV upwards at time  $t = 1.6 \times 10^7$  s.

In our model, the plerion is radiating  $3 \times 10^{39}$  erg s<sup>-1</sup>; this is definitely less than the present bolometric luminosity,  $\sim 2 \times 10^{41}$  erg s<sup>-1</sup>, so an additional energy source must be active. The observed decline, 0.009 mag per day, is a strong indication that <sup>56</sup>Co is providing the extra power. At the quoted rate one will have to wait for one and half years before the two inputs become comparable; if the envelope is severely fragmented, only a fraction of the pulsar power is intercepted and reprocessed by the ejecta, so that the bolometric light curve will be dominated by the pulsar at a still later time.

The average value of the nebular magnetic field turns out to be about 0.5 G. In such a field, electrons radiating at  $10^{18}$  Hz propagate for less than  $10^{14}$  cm. This is consistent with the limits on the fragments' size deduced from the variability time scale. Note however that most of the time the X-ray emission is in a high state; this implies that the covering fraction is small, and that the individual fragments have a larger column density than a uniform shell containing the same total mass. If the radioactive <sup>56</sup>Co is well mixed within the fragments, this could explain why we do not observe a larger flux of comptonized X-rays or a larger flux of unscattered  $\gamma$ -ray lines.

According to our model, the radioemission expected from SN1987A is of the order of 25 mJy, but note that free-free opacity at radio wavelengths is much more severe than photoelectric opacity at X-ray energies, and the latter may become negligible long before the former.

In conclusion, we stress the main observational consequences of our model: (1) a secular X-ray light curve characterized by a slight flux increase (4% per month at present); (2) the existence of flux dips superimposed on the secular behaviour; (3) the possible emergence of a radio source in the near future. Furthermore, we note that a contribution to the X-ray flux could arise directly in the magnetosphere of the neutron star and be pulsed. If a periodic component could be found, this would open new perspectives in the study of the early life of pulsars.

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*Note added in proof:* A rapid increase in the X-ray flux from SN 1987A has been reported in January 1988<sup>15</sup>. This indicates again that the emitting region is considerably smaller than the present size of the supernova envelope and thus argues against the radioactivity model. In our interpretation such an outburst may arise either from a sudden further decrease of the obscuration or from a flare in the magnetosphere of the neutron star which has not yet adjusted to an equilibrium state.

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# Crystal structure of the high-temperature superconductor $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$

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High-temperature superconductivity has gained renewed attention following recent reports of the onset of superconductivity above 100 K in oxide systems containing copper, alkaline earths and bismuth<sup>1</sup> or thallium<sup>2-4</sup>. The superconducting oxide in the Bi-Sr-Ca-Cu-O system has been identified as  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$ , and its structure comprises pairs of  $\text{CuO}_2$  sheets, interleaved by Ca(Sr), alternating with double bismuth-oxide layers<sup>5-7</sup>. Hazen *et al.*<sup>8</sup> have identified two new superconducting phases,  $\text{Tl}_2\text{Ba}_2\text{Ca}_2\text{Cu}_3\text{O}_{10}$  (2223) and  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$  (2212), both with tetragonal or pseudotetragonal structures that are probably related to the structure of  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$ . Here we present X-ray diffraction data for a single crystal of the 2212 phase, which has an onset temperature of ~112 K. The crystal structure, based on a body-centred tetragonal cell with  $a = 3.8550(6)$  Å and  $c = 29.318(4)$  Å (space group  $I4/mmm$ ), is essentially the same as that of  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$  and comprises double copper-oxygen sheets alternating with double thallium-oxygen layers. There are  $\text{Ca}^{2+}$  cations between adjacent Cu-O sheets and  $\text{Ba}^{2+}$  cations between the Cu-O and Tl-O layers. The copper-oxygen chains present in  $\text{YBa}_2\text{Cu}_3\text{O}_7$  are absent in  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$ .

About 100 compositions in the Tl-Ba-Ca-Cu-O system were prepared by reacting  $\text{Tl}_2\text{O}_3$ ,  $\text{CaO}_2/\text{CaCO}_3$ ,  $\text{BaO}_2$  and CuO in various proportions at 850–915 °C in air or in sealed gold tubes for 15 min to 3 h. For sealed-tube reactions,  $\text{CaO}_2$  and  $\text{BaO}_2$  were used as starting reagents. Powder X-ray diffraction revealed that most products were mixtures containing mainly two phases, with characteristic layer spacings ( $d$  values) of ~14.7 and ~18 Å. Hazen *et al.*<sup>8</sup> suggested that these two characteristic reflections arose from the 2212 and 2223 phases, respectively.

Flux exclusion measurements showed superconductivity in our samples with onset temperatures ranging from 100 to 127 K; the sample with an onset temperature of 127 K is poorly crystalline. The presence of a 2223 phase is revealed by a reflection at a  $d$ -value close to 18 Å. A single-phase powder of this material has been prepared and a neutron diffraction study is in progress.

The 2212 phase appears in most of our preparations. The highest transition temperatures are observed when the samples are partially melted at >900 °C. The temperature range in which samples can be prepared without decomposition to barium cuprate phases is very narrow.

Single crystals of the 2122 superconducting phase were grown from a copper-rich melt with oxides in the molar ratio 2:1:1:3 (Tl:Ba:Ca:Cu) in a sealed gold tube. The mixture was heated to 900–920 °C, held for 1 h and cooled at 2° min<sup>-1</sup>. Plate-like crystals found in the melt were mechanically separated and used for further characterization and structure determination. Flux exclusion measurements on the single crystals revealed a sharp superconducting transition at  $T_c \approx 110$  K (Fig. 1). Figure 2 shows the resistivity plot for a polycrystalline sample of  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$ , which also contained minor amounts of other phases.

Figure 3 shows the morphology of the  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$  plates. In contrast to  $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ , these particles are not mica-like, and do not have a tendency to delaminate. Transmission electron microscopy reveals that the material contains defects such as stacking faults and dislocations.

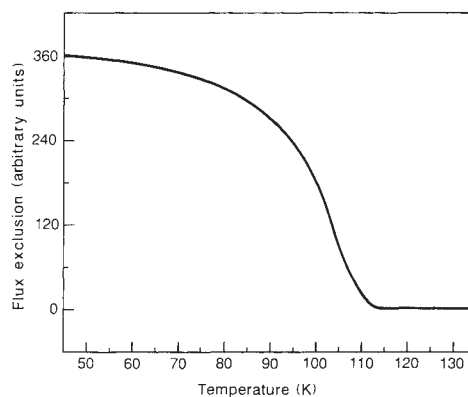


Fig. 1 Magnetic flux exclusion of a few randomly oriented single crystals of  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$  superconductor, measured by an a.c. susceptometer.

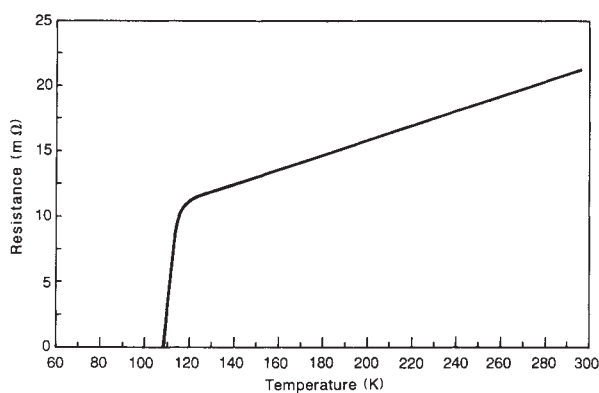


Fig. 2 Resistance versus temperature for polycrystalline  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$ .

The single-crystal X-ray diffraction data are summarized in Table 1. A black platy crystal suitable for X-ray diffraction was selected from an examination of axial rotation photographs and theta-scans, as some crystals showed incommensurate multiple diffraction along the plate axis—potentially a result of intergrowth with a second phase. From the similarity of the lattice constants to those of previously reported  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$ <sup>5</sup>, a primitive data set was collected in a  $5.44 \times 5.44 \times 29.3$  Å cell in anticipation of the substructure isomorphism. Unlike the bismuth plates, the thallium crystals did not display the  $a$ -axis satellite modulation, and owing to the absence of  $F$ -centred violations, we transformed the lattice to the body-centred  $3.86 \times 29.3$  Å tetragonal cell.

An entire sphere of intensity data collected at room temperature was reduced to structure factors in the usual fashion, corrected for absorption, transformed and averaged in  $I4/mmm$  ( $R_{\text{merge}} = 0.021$  for 223 duplicates) to yield 176 reflections for the analysis. A second larger crystal with lattice constants  $a = 3.85$ ,  $c = 29.36$  Å resulted in 208 averaged reflections. The heavy-atom positions were indicated from phases obtained by the direct method (using the computer program Multan 80; P. Main *et al.*, preprint, University of York), and the complete structure was determined with the usual combination of structure factor, Fourier synthesis and least-square refinements.

The crystallographic asymmetric unit consists of the four metal atoms and three unique oxygen atoms, all on special positions (see Table 1a). The resulting coordination geometries are square-planar Cu (with a weak square-pyramidal oxygen interaction), cubic Ca, octahedral Tl and nine-coordinate Ba

**Table 1** Crystallographic data for  $\text{Ti}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$ 

| (a) Positional and thermal parameters*          |      |           |               |                  |                     |                 |                 |                 |                 |                 |                 |
|-------------------------------------------------|------|-----------|---------------|------------------|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Atom                                            | Site | x         | y             | z                | B (Å <sup>2</sup> ) | B <sub>11</sub> | B <sub>22</sub> | B <sub>33</sub> | B <sub>12</sub> | B <sub>13</sub> | B <sub>23</sub> |
| Tl(1)                                           | 4e   | 0.50      | 0.50          | 0.21359(2)       | 1.7(1)              | 2.07(2)         | 2.07            | 0.88(2)         | 0.00            | 0.00            | 0.00            |
| Ba(1)                                           | 4e   | 0.00      | 0.00          | 0.12179(3)       | 0.8(1)              | 0.55(2)         | 0.55            | 1.15(3)         | 0.00            | 0.00            | 0.00            |
| Cu(1)                                           | 4e   | 0.50      | 0.50          | 0.0540(1)        | 0.4(1)              | 0.14(3)         | 0.14            | 1.06(6)         | 0.00            | 0.00            | 0.00            |
| Ca(1)                                           | 2a   | 0.00      | 0.00          | 0.00             | 0.6(1)              | 0.4(1)          | 0.4             | 1.0(1)          | 0.0             | 0.0             | 0.0             |
| O(1)                                            | 8g   | 0.00      | 0.50          | 0.0531(2)        | 0.8(1)              | 0.4(2)          | 0.7(2)          | 1.4(2)          | 0.0             | 0.0             | 0.0             |
| O(2)                                            | 4e   | 0.50      | 0.50          | 0.1461(3)        | 1.5(2)              | 2.0(3)          | 2.0             | 0.7(3)          | 0.0             | 0.0             | 0.0             |
| O(3) <sup>†</sup>                               | 16n  | 0.604(9)  | 0.50          | 0.2815(7)        | 3.9(11)             | 7.0(32)         | 2.1(11)         | 2.5(7)          | 0.0             | 0.0(10)         | 0.0             |
| (b) Interatomic distances (Å) and angles (deg.) |      |           |               |                  |                     |                 |                 |                 |                 |                 |                 |
| Cu(1)–O(1)                                      |      | 1.9277(3) | (×4)          | Ba(1)–O(1)       |                     | 2.788(4)        | (×4)            |                 |                 |                 |                 |
| Cu(1)–O(2)                                      |      | 2.699(10) | (×1)          | Ba(1)–O(2)       |                     | 2.818(3)        | (×4)            |                 |                 |                 |                 |
| Cu(1)–Cu(1)                                     |      | 3.166(3)  | (inter-sheet) | Ba(1)–O(3)       |                     | 2.864(21)       | (×1)            |                 |                 |                 |                 |
| Tl(1)–O(2)                                      |      | 1.978(10) | (×1)          | O(1)–Cu(1)–O(1)  |                     | 178.4(4)        | (×2)            |                 |                 |                 |                 |
| Tl(1)–O(3)                                      |      | 2.031(21) | (×1)          | Cu(1)–O(1)–Cu(1) |                     | 178.4(4)        | (×1)            |                 |                 |                 |                 |
| Tl(1)–O(3)                                      |      | 2.462(22) | (×2)          | O(1)–Cu(1)–O(1)  |                     | 89.99(1)        | (×4)            |                 |                 |                 |                 |
| Tl(1)–O(3)                                      |      | 3.027(28) | (×2)          | O(1)–Cu(1)–O(2)  |                     | 90.8(2)         | (×4)            |                 |                 |                 |                 |
| Ca(1)–O(1)                                      |      | 2.478(4)  | (×8)          |                  |                     |                 |                 |                 |                 |                 |                 |

Tetragonal: space group  $I4/mmm$  (no. 139);  $a = 3.8550(6)$ ,  $c = 29.318(4)$   $\text{\AA}$ . Crystal dimensions,  $0.07 \times 0.05 \times 0.01$  mm; formula weight, 978.6; calculated density,  $7.46 \text{ g cm}^{-3}$ ; CAD4 diffractometer, ambient temperature, Mo  $K\alpha$  radiation, graphite monochromator; scan mode,  $\omega$ ;  $2\theta = 0$ – $60^\circ$ , full sphere;  $\mu = 515.5 \text{ cm}^{-1}$ ; analytical absorption correction; transmission factors, 0.113–0.606; extinction parameter,  $3.9(4) \times 10^{-5}$  mm; 2562 reflections (176 independent,  $I > 3\sigma$ ); data/parameters = 6.3;  $R = 0.018$ ,  $R_w = 0.018$ .

\* All atoms refined with anisotropic thermal parameters. (The form of the anisotropic displacement parameter is:  $\exp[-0.25(B_{11}h^2a^{*2} + 2(B_{12}hka^*b^* + \dots))]$ ;  $B$  is the equivalent isotropic thermal parameter.

<sup>†</sup> This atom refined off the ideal site ( $\frac{1}{2}, \frac{1}{2}, z$ ).

(face-capped cubic antiprism). Thus, the basic bi-capped Cu–Ca–Cu bilayer structure found in the  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$  analysis is again substantiated here, but shifted by  $(0, \frac{1}{4}, \frac{1}{4})$  because of the different space-group origins.

Refinement of the model was achieved by full-matrix least-squares analysis using neutral-atom scattering-factor curves, with anomalous scattering terms for the metal ions, a term for isotropic secondary extinction, and anisotropic thermal motion for all atoms. Occupation factors for the metal atoms were found to be high for Ca and low for Tl, suggesting partial occupancy of Tl on the Ca site. A comparison of ionic radii<sup>9</sup> (Tl, 0.98  $\text{\AA}$ ; Ca, 1.12  $\text{\AA}$ ; Ba, 1.42  $\text{\AA}$ ) shows this to be a reasonable assumption. Assuming that all of the metal atom sites are occupied, a stoichiometry of  $(\text{Ti}_{1.8}\text{Ca}_{0.2})\text{Ba}_2(\text{Ca}_{0.9}\text{Ti}_{0.1})\text{Cu}_2\text{O}_8$  is approximated from refinement of the site multiplier as a function of the ratio of the Tl and Ca scattering-factor curves. It is difficult to say, however, whether the Tl sheets are fully occupied. The refinement also indicated a high thermal dispersion for one oxygen atom, O(3), and reducing its symmetry resulted in a 0.41- $\text{\AA}$  displacement of this atom from the ideal  $4mm$  site, and a local Tl distortion. The largest correlation in the refinement, 0.66, occurred between the scale factor and  $B_{11}$  of the Tl atom, and the largest peak in a difference Fourier map was 0.03 electrons per  $\text{\AA}^3$ , located 1.1  $\text{\AA}$  from the calcium. The refinement of the data from the second crystal yielded essentially the same partial occupancies and distortions, with final residuals  $R = 0.054$  and  $R_w = 0.051$ .

The crystal structure of  $\text{Ti}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$  is very closely related to that of the recently reported  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$ <sup>5–7</sup>. Double sheets of corner-sharing square-planar ( $C_{4v}$ )  $\text{CuO}_4$  groups are oriented parallel to the (001) plane (Fig. 4). Cu–O bond distances within the sheets are 1.928  $\text{\AA}$  and are slightly longer than the corresponding bonds in the bismuth phase, 1.91  $\text{\AA}$ . There are additional oxygen atoms located above and below the double Cu–O sheets, which are located 2.70  $\text{\AA}$  from the copper atoms. The equivalent copper–oxygen distance in the bismuth material is considerably shorter,  $\sim 2.5$   $\text{\AA}$ . There are no oxygen atoms between the double Cu–O sheets of either the Tl or Bi compounds. The sheets are separated by 3.2  $\text{\AA}$  (Cu–Cu distance),

with calcium (and possibly some thallium) ions coordinated to eight oxygen atoms with  $D_{4h}$  site symmetry and a Ca–O distance of 2.48  $\text{\AA}$ . The structural features described above are also seen in the  $\text{RBa}_2\text{Cu}_3\text{O}_7$  superconducting phases, where the copper–oxygen sheets are separated by 3.3  $\text{\AA}$  with trivalent rare-earth cations.

Barium ions reside just above and below the Cu–O double sheets, in nine-coordination with oxygen. Analogous to  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$ , the Ba–Cu–Ca–Cu–Ba slabs in  $\text{Ti}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$  alternate with double thallium–oxygen sheets, giving a layer repeat sequence of ...Ti–Ti–Ba–Cu–Ca–Cu–Ba... along the  $c$  direction. Thallium bonds to six oxygen atoms in a distorted octahedral arrangement (see Fig. 4). In contrast with the bismuth material, there is no obvious disorder in the double thallium–oxygen layers. But a subtle disorder may be suggested by the relatively larger basal ( $B_{11} = B_{22}$ ) thermal parameters for Tl (Table 1a), coupled with the large anisotropic  $B_{11}$  thermal parameter for O(3). This could be evidence for the existence of thallium-deficient sheets, in which the oxygen atoms within the sheets are statistically positioned around the  $(\frac{1}{2}, \frac{1}{2}, z)$  (4e) site in order to form shorter bonds to thallium. The average Tl–O bond distance, with O(3) on the  $(\frac{1}{2}, \frac{1}{2}, z)$  sites, is 2.48  $\text{\AA}$ , significantly longer than the sum of ionic radii<sup>9</sup>, 2.28  $\text{\AA}$ . The O(3) site disorder results in two Tl–O bonds of 2.4  $\text{\AA}$  and two of 3.03  $\text{\AA}$ . Low-temperature single-crystal X-ray and powder neutron diffraction studies of the 2212 and 2223 (Ti:Ba:Ca:Cu) and 221 (Ti:Ba:Cu) phases are in progress.

Copper–oxygen sheets where the copper is in essentially square-planar coordination are present in all of the known high- $T_c$  superconductors. This includes  $\text{La}_{2-x}\text{A}_x\text{CuO}_4$ , where A is an alkaline earth (Ba, Sr or Ca)<sup>10</sup> or Na<sup>11</sup>,  $\text{Ti}_2\text{Ba}_2\text{CuO}_6$  and  $\text{Bi}_2\text{Sr}_2\text{CuO}_6$ <sup>13</sup>,  $\text{RBa}_2\text{Cu}_3\text{O}_7$ <sup>12</sup> where R is nearly any rare earth, and the  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$  and  $\text{Ti}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$  phases. The compounds with the lower transition temperatures,  $\text{La}_{2-x}\text{A}_x\text{CuO}_4$  (20–40 K) and  $\text{Bi}_2\text{Sr}_2\text{CuO}_6$  (0–20 K), contain single sheets where there are, in addition, two more oxygen atoms bound to copper, forming a distorted octahedron. But we have just determined the structure of  $\text{Ti}_2\text{Ba}_2\text{CuO}_6$  from single-diffraction data<sup>13</sup>, and find that it contains single Cu–O



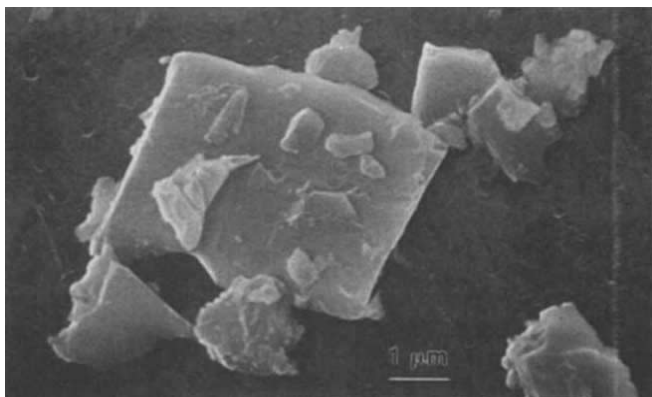


Fig. 3 Morphology of crystals of  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$ .

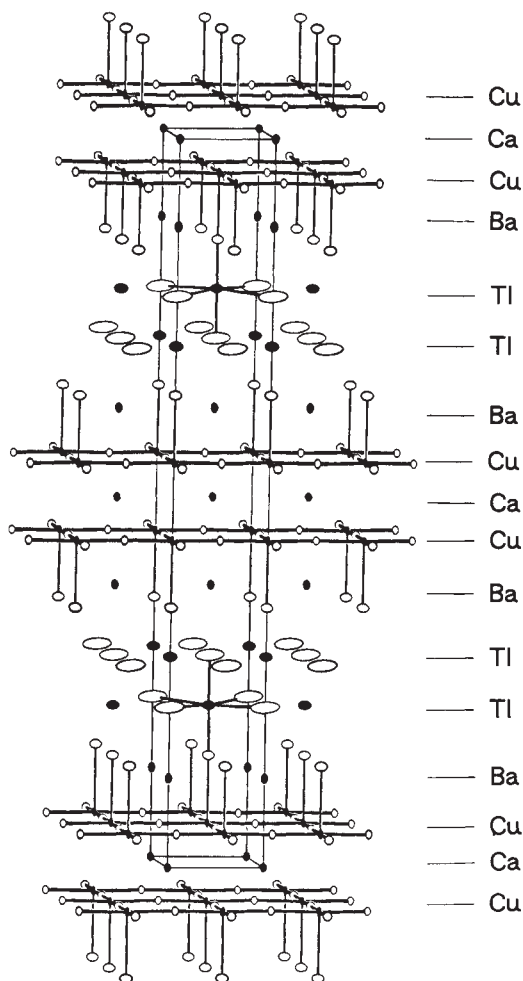


Fig. 4 Crystal structure of  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$ . Metal atoms are shaded and Cu-O bonds are shown. The O(3) atoms with highly anisotropic temperature factors are positioned on the ideal  $(\frac{1}{2}, \frac{1}{2}, z)$  site.

sheets as well as the double Tl-O sheets found in  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$ . The  $\text{Tl}_2\text{Ba}_2\text{CuO}_6$  material is a superconductor with apparent zero resistivity at 83 K<sup>13</sup>. In the other high- $T_c$  materials, double Cu-O sheets are present, in which the copper has a fifth oxygen atom with a longer Cu-O distance to form a square pyramid around copper. The degree of planarity for the copper-oxygen sheets increases in the order:  $\text{RBa}_2\text{Cu}_3\text{O}_7 < \text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8 < \text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$ . A measure of this planar-

ity is the distance from the sheet copper to the average plane of the four oxygen atoms within the sheets. The respective distances for  $\text{RBa}_2\text{Cu}_3\text{O}_7$ ,  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8$  and  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$  are 0.27, 0.09 and 0.03 Å. This correlates with the length of the fifth Cu-O bond perpendicular to the sheets: 2.3, 2.5 and 2.7 Å, respectively.

The differences in the nature of the Bi-O and Tl-O layers are likely to be responsible for the dissimilar morphologies of the two materials. The mica-like nature of the bismuth compound is probably due to the weak inter-sheet Bi-O bonds ( $\sim 3.3$  Å in  $\text{Bi}_2\text{Sr}_{3-x}\text{Ca}_x\text{Cu}_2\text{O}_8^{5-7}$  and  $\text{Bi}_2\text{Sr}_2\text{CuO}_6^{13}$ ). Cleavage between the two adjacent Bi-O layers would yield charge-neutral segments. For comparison, note that the inter-sheet Tl-O bonds in  $\text{Tl}_2\text{Ba}_2\text{CaCu}_2\text{O}_8$  are much stronger (only 2.0 Å long), resulting in a lesser tendency to delaminate.

The Cu-O chains present in  $\text{YBa}_2\text{Cu}_3\text{O}_7$  and thought by some to be very important for high- $T_c$  superconductivity are not present in either the bismuth or thallium compounds. The common structural features shared by the new high- $T_c$  oxides lead to the following surviving guidelines in the search for new superconducting oxides: (1) they should be based on Cu-O chemistry; (2) they should contain copper in a mixed-valence state (2+ and 3+), and (3) in essentially square-planar units, which share corners to form infinite sheets; (4) they should be anisotropic in structure and properties.

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## Growth of superconducting single crystals in the Bi-Sr-Ca-Cu-O system from alkali chloride fluxes

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The discovery of superconductivity at temperatures above the boiling point of nitrogen has led to intense interest in the physics and chemistry of materials exhibiting this behaviour. Although some experiments can be performed on sintered polycrystalline samples, large single crystals are important for many measurements of physical properties, including investigations of the relationship between structural parameters and superconducting properties. Like its predecessors  $\text{YBa}_2\text{Cu}_3\text{O}_7$  (refs 1-4) and

$\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$  (ref. 5), the recently discovered 84-K bismuth-strontium-calcium cuprate superconductor<sup>6-9</sup> melts incongruently, and crystals have been grown from eutectic melts<sup>10</sup>. This technique yields crystals large enough for some experiments, but is generally difficult to control, placing limits on the size of crystals that can be grown. In addition, the crystals must be mechanically separated from the melt. Here we report the growth of single crystals of  $\text{Bi}_{2.2}\text{Sr}_2\text{Ca}_{0.8}\text{Cu}_2\text{O}_{8+\delta}$  from alkali chloride fluxes. The crystals are superconductors, showing large Meissner effects and zero-resistance transitions above 80 K. Flux growth is a standard high-temperature solution growth technique, and should allow crystal size and quality to be controlled by parameters such as equilibration temperature, cooling rate and melt composition. The fluxes reported here are easily washed from the crystals.

Pre-reacted Bi-Sr-Ca-Cu-O mixtures were prepared from high-purity  $\text{Bi}_2\text{O}_3$ ,  $\text{SrCO}_3$ ,  $\text{Ca(OH)}_2$  and  $\text{CuO}$  by slow heating to 800–850 °C with intermediate grinding steps. These pre-reacted starting materials were generally not single-phase. Charges consisting of 10–50 wt% Bi-Sr-Ca-Cu-O mixtures thoroughly mixed with NaCl, KCl or other alkali halide salt or salt mixtures were placed in crucibles, heated above the melting temperature of the salt or salt mixture, and cooled at rates of 1–10 °C h<sup>-1</sup>. In a typical experiment, a 20 wt% mixture of  $\text{BiSrCaCu}_2\text{O}_x$  in KCl was heated rapidly to 875 °C, held at that temperature for 1 h, and then cooled at 10 °C h<sup>-1</sup> to 760 °C. Crystals grew mainly on top of the salt, although crystals were also observed within the solidified charge. Crystals were separated mechanically from the solidified melt, and adherent salt was easily washed from the crystal with no apparent loss of superconductivity in the sample. Energy-dispersive spectroscopic analysis showed no detectable levels of alkali metal or chloride in the crystals.

Several types of crucible were used to contain the melts. Platinum crucibles reproducibly yielded high-quality crystals with no evidence of platinum incorporation. Superconducting crystals were also obtained using high-density, high-purity (Coors) alumina crucibles, but results proved less reproducible. Among other fluxes that were explored for the growth of  $\text{Bi}_{2.2}\text{Sr}_2\text{Ca}_{0.8}\text{Cu}_2\text{O}_{8+\delta}$  crystals, neither sodium tetraborate nor lithium metaborate melts yielded any crystals, even at high solute concentrations. Lead oxide in gold crucibles does yield some superconducting crystals, but container problems are encountered, with the gold showing some signs of attack. When platinum crucibles are used with lead oxide, new alkaline-earth platinum cuprate crystals are obtained (L. F. Schneemeyer, S. A. Sunshine and T. Siegrist, in preparation).

The crystals of  $\text{Bi}_{2.2}\text{Sr}_2\text{Ca}_{0.8}\text{Cu}_2\text{O}_{8+\delta}$  form with a plate-like morphology. Crystals are often bar-shaped, at present  $\geq 1$  mm wide and several millimetres in length, with thicknesses of a few micrometres. In many cases, the material grows in the form of platelets with surface areas of the order of a few square centimetres, and the *c*-axis normal to the platelet face. Perhaps partly owing to their small thickness, the platelets show great flexibility or apparent ductility. We find, however, that the structural quality and phase purity of the bar-shaped crystals are superior to those of the platelets. Figure 1 shows some as-grown platelets. Growth parameters such as the cooling rate strongly influence crystal size, with slower growth rates producing larger crystals, as expected. Additional work is necessary to establish optimal melt compositions, cooling rates, and soak times and temperatures. Characterization of crystals of the 84-K superconductor by X-ray diffraction is reported in ref. 8.

Crystals were screened for superconductivity by monitoring their magnetic-field-dependent microwave absorption near  $H = 0$ . Figure 2 shows logarithmic plots for two samples, both of which display an absorption onset at 115 K and a major absorption increase near 90 K. At all temperatures, absorption was anisotropic and greatest with the field parallel to the *c*-axis. This technique is sufficiently sensitive that crystals weighing only a few microgrammes can be characterized in minutes, but there

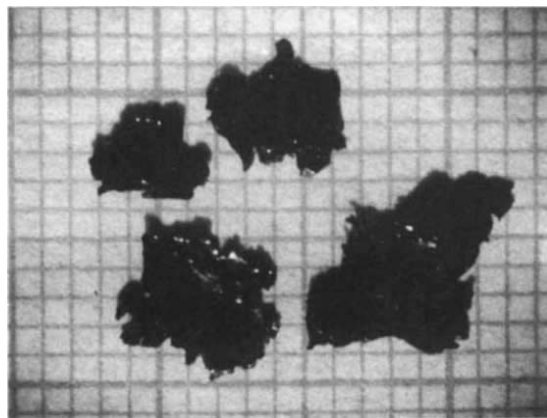


Fig. 1 Platelets of  $\text{Bi}_{2.2}\text{Sr}_2\text{Ca}_{0.8}\text{Cu}_2\text{O}_{8+\delta}$ . Grid spacing, 1 mm.

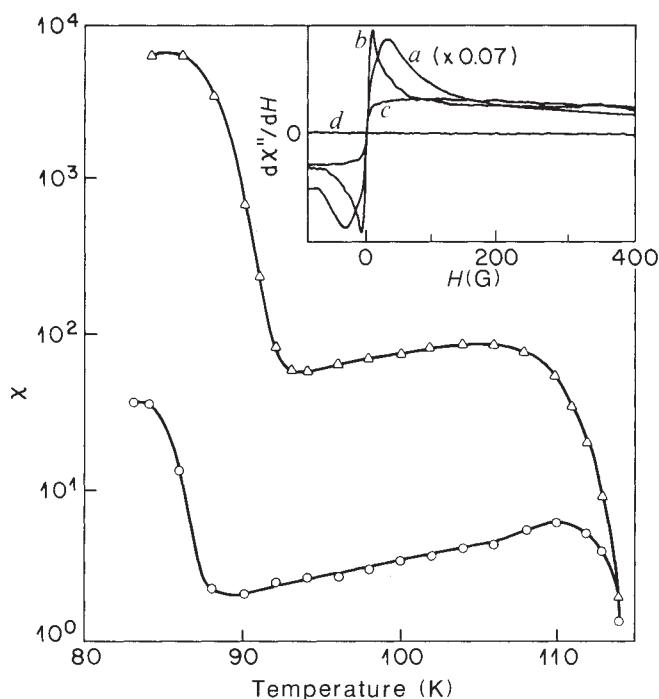


Fig. 2 Relative field-dependent 9-GHz absorption for two crystals. The inset shows typical experimental results for one crystal (triangles in main figure) at 86 K (a), 93 K (b), 110 K (c) and 15 K (d). The points plotted in the main figure were obtained by integration of such data.  $\chi''$  is proportional to absorption and is plotted in arbitrary units.

may not be a direct relationship between absorption and the amount of superconducting phase present.

Superconductivity was further confirmed by resistivity and d.c. susceptibility measurements, as reported in ref. 8. Figure 3 shows the temperature dependence of the resistance for one sample, illustrating a number of characteristic features. Here, the resistance is monitored by the application of a low-frequency (21 Hz) alternating current across two contacts to the sample, as indicated in the inset, and monitoring the voltage on the other contacts with a phase-sensitive detector. The contacts were made using silver paste fired at 700–800 °C, and were typically non-rectifying with a specific contact resistance of  $1.5 \times 10^{-3} \Omega \text{ cm}^2$ .

The sample is significantly anisotropic, as shown by the marked difference in the temperature dependence of the resistivity  $\rho(T)$  for current flow in the two directions. This is seen



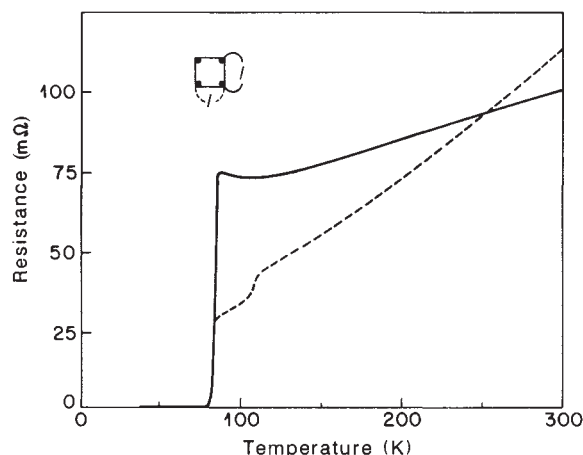


Fig. 3 Temperature dependence of the resistance of a  $\text{Bi}_{22}\text{Sr}_2\text{Ca}_{0.8}\text{Cu}_2\text{O}_{8+\delta}$  single crystal measured in two directions in the  $a$ - $b$  plane. Sample dimensions,  $\sim 1 \times 1 \times 0.001$  mm. The inset shows the two alternatives for the current connections, corresponding to the two curves; the voltage was measured using the free contacts.

both in the high-temperature ( $T > 120$  K) and low-temperature regimes. The anisotropy may be intrinsic, corresponding to the observed structural anisotropy in the  $a$ - $b$  plane<sup>8</sup>, or may result from microscopic inhomogeneity. As we lack a definitive interpretation of the anisotropy, we have neither calculated the temperature dependence of the geometric average resistivity,  $(\rho_x \rho_y)^{1/2}$ , nor attempted to deconvolve the measurements into independent  $\rho_x(T)$  and  $\rho_y(T)$  components. We note, however, that the application of the van der Pauw-Price technique to the data at 300 K yields a value  $(\rho_x \rho_y)^{1/2} = 40 \mu\Omega \text{ cm}$ , assuming a thickness of  $1 \mu\text{m}$ .

The 10–90% transition width in this sample, measured in both directions, is  $\sim 1.5$  K for the transition centred at 81.5 K. This is substantially sharper than previously reported<sup>8</sup>. Zero resistance was achieved at 80 K, although in other samples the zero-resistance temperature varied from 79 to 84 K. The transition starting at 109 K, seen in this sample in one direction only, is also ascribed to superconductivity.

The effect of increasing the measuring current was studied over the range  $1$ – $100 \text{ A cm}^{-1}$  (calculated using the entire sample cross-section), and the 109-K transition shifted by less than 1 K over this range. We note that a current density of  $\sim 1,000 \text{ A cm}^{-2}$  is sufficient to shift the zero-resistance point by 1 K (further details on transport and magnetic critical currents will be reported elsewhere), and conclude by analogy that a substantial fraction of the material must become superconducting at 109 K. Yet no sign of this transition is seen in the other direction.

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## The transient detection microscope

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We describe a microscope that preferentially displays moving objects. This all-optical device operates in real-time using the nonlinear coupling of optical beams in a photorefractive crystal of barium titanate. A holographic image is continuously written and compared with the real image, and any discrepancy between the two images is immediately visible. The device can be used to highlight moving objects, such as protozoans, while suppressing the image of any stationary objects in the background. The device needs only milliwatts of laser power, and can detect objects moving at velocities of a few micrometres per second.

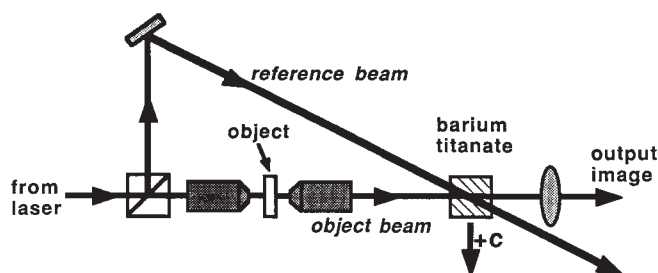
Electronic transient detectors have been used by airport controllers to detect moving airplanes and to eliminate buildings and trees from their television displays. These electronic processors subtract a stored image from the current image serially, pixel by pixel. The transient detection microscope described here optically subtracts two images in parallel, so that the entire image is processed at the same time. Subtraction is accomplished by the nonlinear coupling of two optical beams in a photorefractive crystal, and is analogous to holographic interferometry.

The transient detection microscope uses no electronics; it is a single-task, all-optical processor, which continuously updates the image to be optically subtracted<sup>1</sup>. The hologram of the image, recorded in the barium titanate crystal, is constantly being written and read in real-time, and the old image is continuously subtracted from the new one. A barium titanate crystal is used because it exhibits strong coupling between even weak (microwatt) optical beams, and its time response can be made quite slow (seconds) using low-intensity optical beams.

The transient detector relies on the transfer of energy between two optical beams, as follows. Consider an object-bearing beam (or 'object' beam) and a 'reference' beam intersecting in the barium titanate crystal. If the two beams are coherent, they can produce an optical interference pattern that, by the photorefractive effect, creates a phase hologram inside the crystal<sup>2</sup>. The same beams that write the hologram can also read it. Consequently, a fraction of the reference beam will be diffracted by the hologram into the same direction as the transmitted object beam. (Similarly, a fraction of the object beam will be diffracted by the hologram into the direction of the transmitted reference beam.) The diffracted reference beam receives a  $+90^\circ$  phase shift upon reflection from the phase hologram, and another  $+90^\circ$  phase shift due to the diffusive writing mechanism inside the barium titanate crystal (that is, there is a  $90^\circ$  phase shift between the interference pattern of the light beams and the resulting refractive-index grating formed inside the crystal). The net phase shift between the diffracted reference beam and the transmitted object beam will be  $180^\circ$ , which causes the two beams to interfere destructively. (Conversely, the diffracted object beam receives a net phase shift of  $0^\circ$ , and so it interferes constructively with the transmitted reference beam.) The net effect is that the object beam exits the crystal greatly diminished in intensity, with its energy having been transferred to the reference beam. The intensity of the transmitted object beam,  $I'_o$ , is given by<sup>3,4</sup>

$$I'_o = \frac{I_o + I_r}{1 + (I_r/I_o) \exp(\Gamma s)} \quad (1)$$

where  $I_o$  and  $I_r$  are the intensities of the transmitted object and reference beams in the absence of coupling,  $s$  is the interaction length in the crystal, and  $\Gamma$  is the photorefractive coupling constant, which depends on the wavelength, polarization, and



**Fig. 1** The incident laser beam is split into an object beam and a reference beam, which intersect in a photorefractive crystal of barium titanate, with the *c*-axis oriented as shown. The transmitted object beam contains the images of only the moving objects on the microscope slide.

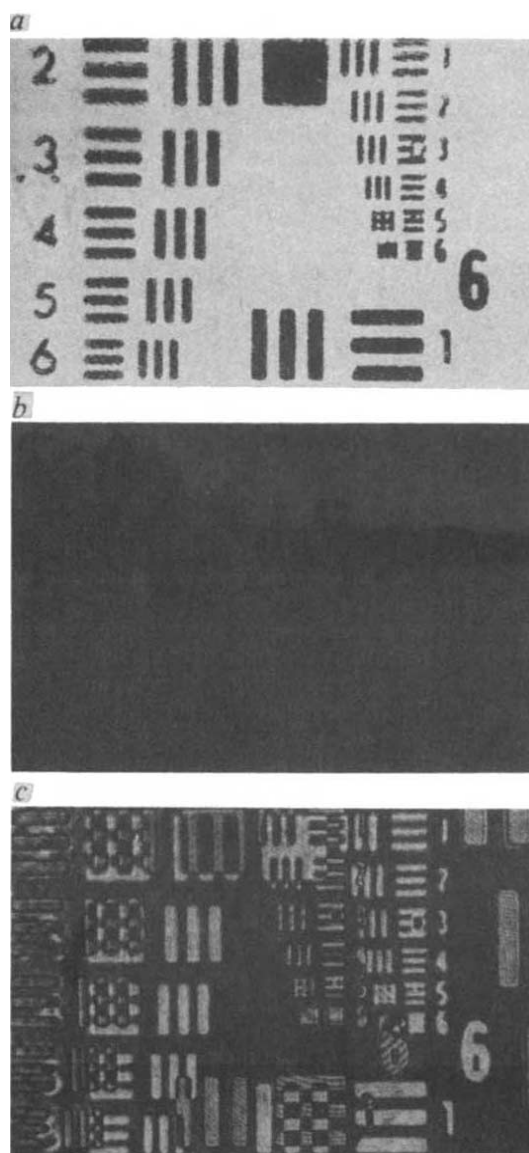
angle between the two beams, as well as the type and orientation of the photorefractive crystal<sup>2</sup>. From equation (1), the fraction  $I'_o/I_o$  of the object beam intensity emerging from the crystal is

$$I'_o/I_o \approx \exp(-\Gamma s) \quad (2)$$

for  $I_o \ll I_r$ . For the experiments reported here, the coupling strength is  $\Gamma s = 3.4$ , so that the object beam loses about 97% of its energy to the reference beam. The photorefractive hologram requires a finite time  $\tau$  to be written or erased. If either optical beam changes in a time short compared with  $\tau$ , the destructive interference described above is no longer optimized, and the image will become immediately visible. Note that this enhancement of moving objects occurs instantly, and is not limited by the response time  $\tau$ . In particular, if just a portion of the object beam changes rapidly compared with  $\tau$ , that portion will emerge from the crystal with its intensity enhanced, while the rest of the image will be depleted, as before<sup>5</sup>. Thus any moving or changing images in the object beam will be highlighted against a dark, stationary background.

The device resembles an ordinary transmission microscope with a microscope objective lens and an eyepiece or camera for viewing the real image. However, here the illumination is by laser light, and a barium titanate crystal is located between the objective and eyepiece. As shown in Fig. 1 the object beam from an argon-ion laser is focused by a  $\times 10$  microscope objective to a  $100 \mu\text{m}$  diameter spot, which illuminates the object. A second  $\times 10$  microscope objective collects the light, and forms a real image of the object in a barium titanate crystal. A second laser beam (the reference beam), coherent with and considerably more intense than the object beam, is also directed into the crystal. The ferroelectric axis of the barium titanate crystal is oriented so that energy is transferred from the object beam into the reference beam. The portion of the object beam transmitted through the crystal is viewed either with an eyepiece or with a television camera.

Figure 2 shows the image of a resolution target with and without the transient detector. Without the reference beam, the apparatus is an ordinary microscope, and the entire image is visible, as seen in Fig. 2a. The resolution is about  $5 \mu\text{m}$ . With the reference beam, transient detection is activated, and the image appears almost black, as in Fig. 2b, because nothing in the object beam is changing in phase, amplitude or position. However, if the target is suddenly moved, a bright image instantly appears, as seen in Fig. 2c. As the chart has moved from its old position, the device detects the change in the old image and displays it. The chart is also in a new position where it was not before, so the transient detector displays this new image as well. These two images produce the chequerboard pattern seen in Fig. 2c, where horizontal lines have overlapped vertical ones. The transient detector 'tracks' the changing input, updating and rewriting new sets of holograms, as fast as the crystal response time allows.

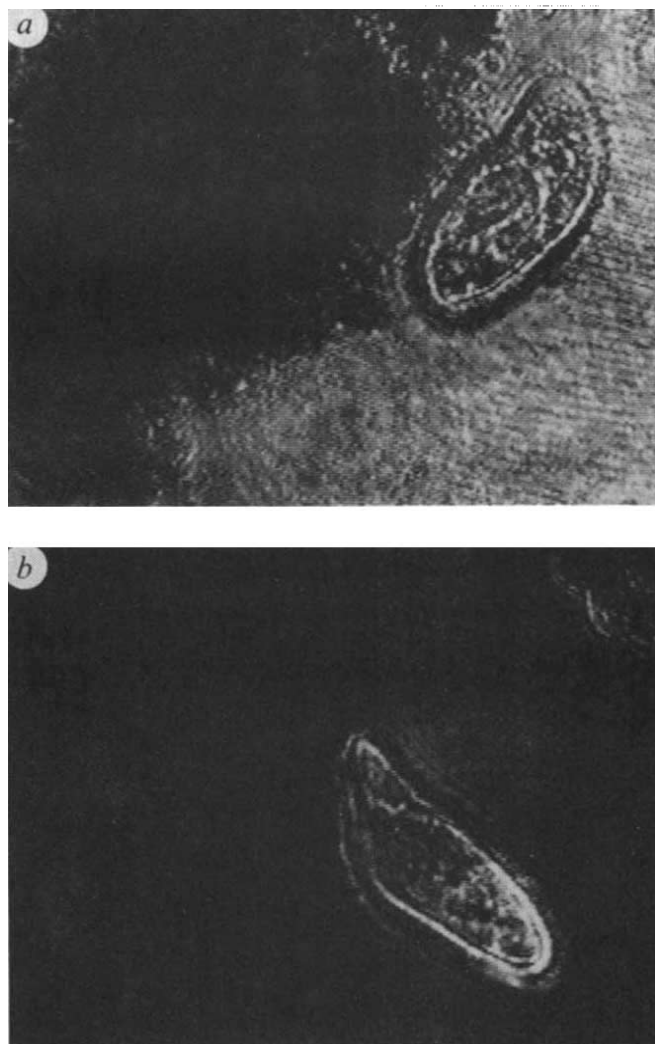


**Fig. 2** *a*, Image of a resolution chart without the reference beam of Fig. 1, i.e. with the transient detector turned off. *b*, Same object, but with the transient detector turned on. The image is almost black, because the object is not moving or changing. *c*, Image obtained immediately after the resolution chart was translated about  $30 \mu\text{m}$  to the left. Note that both the old image and the new image are displayed simultaneously.

In Fig. 3 the transient detector is used to preferentially display the motion of small, self-animated objects found in a local pond. These protozoans, which range in size from  $5$  to  $30 \mu\text{m}$ , can be seen in Fig. 3a, in which the transient detector is turned off; the non-moving algae in the background are also plainly visible. Figure 3b shows the scene a moment later, where now the transient detector has been turned on. The images of any non-moving objects in the scene, including the algae and any stationary protozoans, have been largely removed from the scene by the transient detector. Standing out is the protozoan that happened to be moving when the photograph was taken<sup>6</sup>.

Note that if the input pattern changes too slowly, the transient detector will track the changes, and no output will be produced. Any object must move at least one resolvable spot in a time shorter than the characteristic writing time for the transient detector to 'see' it. For linear motion, the minimum detectable





**Fig. 3** *a*, Image of pond water with the transient detector turned off. Both the stationary algae and the swimming protozoan are visible. *b*, Image obtained with the transient detector turned on: only the moving protozoan is visible.

velocity is determined by the crystal's response time  $\tau$  according to

$$v = d/\tau \quad (3)$$

where  $d$  is the minimum resolvable feature size of the microscope, and  $\tau$  is the hologram writing time, which is a function of the total optical intensity  $I_r + I_o$  in the crystal<sup>2</sup>. Note that a wide range of minimum speeds may be selected by varying the intensity of the reference beam, while leaving the object beam intensity fixed at some level small enough to avoid damaging the sample. The images seen in Figs 2 and 3 were produced with 10 mW in the reference beam focused to a 2-mm diameter spot inside the crystal ( $I_r = 0.3 \text{ W cm}^{-2}$ ) and only  $80 \mu\text{W}$  of object beam power focused to a 0.5-mm diameter spot ( $I_o = 0.04 \text{ W cm}^{-2}$ ). The response time  $\tau$  with this illumination was 0.2 s and the resolution  $d$  was  $5 \mu\text{m}$ , which from equation (3) gives a minimum detectable speed of  $25 \mu\text{m s}^{-1}$ .

Measurements were taken using the various blue and green lines of an argon-ion laser, with similar results, although deep blue light produces higher-contrast images. We also tried the red 633-nm line of a low-power helium-neon laser, using only  $1 \mu\text{W}$  for the object beam. (The corresponding intensity in the sample was  $10^{-2} \text{ W cm}^{-2}$ , which, for comparison, is an order of magnitude less than the intensity of daylight.) We note that the

contrast between the dark background and the brightest images dropped from 30:1 in the blue to 5:1 in the red, owing to the decrease in the strength of the nonlinear optical coupling of the barium titanate crystal at longer wavelengths. The 442-nm line of the helium-cadmium laser would be an ideal light source for transient detection microscopy because of the higher coupling strength<sup>7</sup> and increased resolution possible at this wavelength.

We also point out that it is possible to build an image intensifier from the same geometry as that used for the transient detection microscope, simply by aligning the crystal's  $c$ -axis in the opposite direction to that shown in Fig. 1. Such a device would be the opposite of a transient detector, and would intensify, by the same gain factor  $\exp(\Gamma s)$ , only the images of stationary objects.

We point out some of the difficulties of the present device. First, the crystal must be of very high optical quality or it will distort the transmitted image. This sensitivity to crystal quality can be removed by using a phase-conjugate geometry<sup>1</sup>. Another problem is seen in Fig. 2c, in which two images of the moving resolution chart are seen. Such multiple images will always be present, causing a faint tail to appear behind the image of moving objects. Finally, imaging with coherent light requires dust-free optics to avoid noisy speckle patterns.

Thus we have demonstrated an all-optical device that detects microscopic changes in a scene. The device is a single-task processor that detects any difference between two states of a microscopic sample, using real-time holography in a photo-refractive crystal. Unlike analogous systems, which use spatial light modulators or digital image processors, in this device the object is not partitioned into discrete pixels, so the resolution is not compromised. This device can also function as an all-optical image intensifier for stationary objects, which may prove useful for observing a stationary specimen under extremely subdued light.

**Note added in proof:** A related paper describing temporal differentiation of non-microscopic objects has been recently published<sup>8</sup>.

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## Ionic recognition and selective response in self-assembling monolayer membranes on electrodes

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Communication in living organisms is governed by cell bilayer membranes, which selectively recognize a specific component in the presence of others and accordingly respond. The functioning of such molecular-size barriers involves molecular and quantum processes deriving from a precise, purpose-oriented architecture, and attempts have been made to create artificial supramolecular structures exhibiting similar properties<sup>1–9</sup>. In particular, chemi-

cally modified electrodes, coated with various types of organic layers<sup>10-18</sup>, have been used to control the access of electroactive species from solution, but such systems have so far lacked some of the important features of real, molecular-size membranes. Here we present the first example of an electrode coated with a stable, ion-selective artificial membrane having the thickness of just one molecule, which successfully mimics basic structural and functional principles of the natural bilayer membrane. This monolayer membrane, produced by molecular self-assembly on gold, can recognize a selected metal ion in the presence of other ions, and thus induces a specific electrode response. It consists of synthetic 'receptor sites', designed to impart the desired selectivity, embedded within an inert monolayer matrix which blocks vacant sites on the surface and so prevents the passage of undesired species. The supporting gold electrode permits electrochemical analysis of the membrane structure and performance. Such monolayer membranes may aid the study of elementary charge transfer processes at liquid-solid interfaces, and contribute to future molecular-based technologies.

Impermeable one-molecule-thick barriers for ions and water have recently been produced on gold<sup>19-21</sup> using techniques of monolayer self-assembly<sup>19-25</sup>. Achieving ion selectivity in such systems is more complex, as the 'active' element providing the desired selectivity may not form film structures that are compact enough to prevent leakage of other, undesired species. We show here the feasibility of an approach based upon the use of self-assembled mixed monolayers, containing both 'active' (monolayer-forming ligand) and 'blocking' (surface-sealing long-chain amphiphile) components<sup>25</sup>, so that a specific response for metal ions forming 1:1 complexes with the ligand is achieved.

The ligand used was 2,2'-thiobisethyl acetoacetate,  $S(CH_2CH_2OCOCH_2COCH_3)_2$  (TBEA, Fig. 1), designed and synthesized as 'active' component. The two  $\beta$ -keto ester groups of TBEA form a tetradentate chelating centre, and the sulphur bridge was designed to anchor the ligand to a gold surface<sup>19,20,23,24,26</sup>. Surface-bound tetradentate TBEA is an excellent candidate for the formation of 1:1 complexes with divalent metal ions, such as  $Cu^{2+}$ , but is geometrically unsuited for binding trivalent metal ions, such as  $Fe^{3+}$ , that require octahedral coordination. Therefore, in terms of geometric discrimination (and also electrochemical suitability),  $Cu^{2+}$  and  $Fe^{3+}$  are convenient ionic probes to test the selectivity of the present monolayer-coated electrodes.

Figure 2a shows a cyclic voltammogram of a bare gold electrode in  $H_2SO_4$  solution containing  $Cu^{2+}$  and  $Fe^{3+}$ . The  $Fe^{3+/2+}$  reduction-oxidation peaks are marked with arrows; the small peaks around  $-0.15$  V correspond to underpotential deposition (UPD) of a monolayer of Cu on Au<sup>27</sup> and the peaks at  $-0.55$  V (reduction) and  $-0.40$  V (oxidation) correspond to deposition-dissolution of bulk Cu. Examination of an electrode coated with only TBEA (Fig. 2b) indicates a film structure not sufficiently compact to prevent leakage of  $Fe^{3+}$  through uncovered portions of the electrode. (TBEA was adsorbed on gold by immersion in a solution containing  $3.3 \times 10^{-2}$  M TBEA in bicyclohexyl:chloroform, 4:1 v/v, for 3.5 h; the resulting electrodes are denoted Au/TBEA.) Evidently the introduction of an appropriate 'blocking' element is necessary for the proper functioning of the system. It was expected that addition of a surface-sealing monolayer component to the adsorption solution might result in a continuous mixed monolayer barrier with ion-selective sites embedded within a compact, electrochemically inert matrix. Previous experience<sup>19-21</sup> pointed to *n*-octadecyl mercaptan,  $CH_3(CH_2)_{17}SH$  (OM, Fig. 1) as a suitable such component. Thus mixed monolayer membranes were prepared on gold substrates by co-adsorption of the two components from a solution containing  $2.0 \times 10^{-2}$  M TBEA +  $2.0 \times 10^{-2}$  M OM in bicyclohexyl:chloroform, 4:1 v/v, for 3.5 h. These electrodes are denoted Au/(TBEA+OM).

The performance of the mixed (TBEA+OM) monolayer

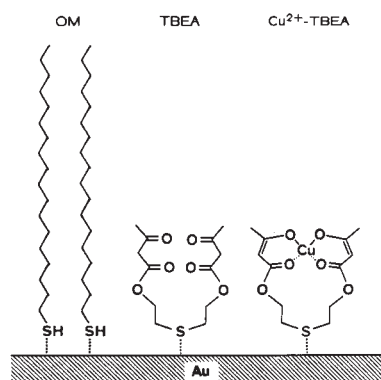


Fig. 1 Schematic representation of TBEA,  $Cu^{2+}$ -TBEA complex, and *n*-octadecyl mercaptan (OM), adsorbed on gold substrate. Note that ligand binds  $Cu^{2+}$  in the enol form upon losing two protons, and thus the complex is neutral. TBEA was prepared by the 4-dimethylaminopyridine (DMAP)-catalysed reaction<sup>29</sup> of 2,2'-thiobisethanol with diketene. Gold electrodes were prepared by sputter-deposition of  $\sim 1,000$  Å gold on glass microscope slides<sup>19,21</sup>, followed by annealing for 15 min at  $420^\circ C$ , which reduces the gold-surface roughness.

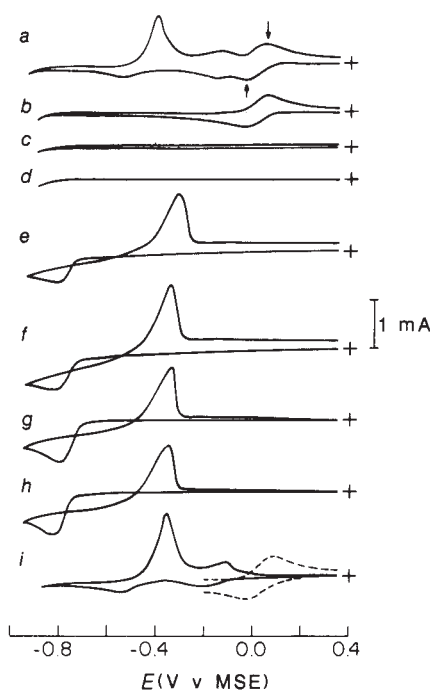
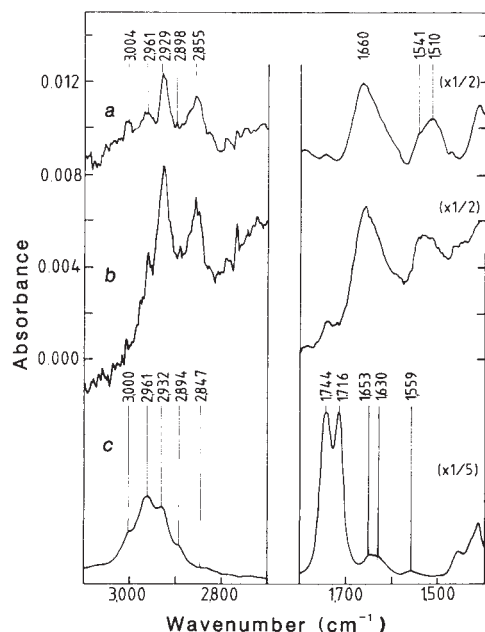


Fig. 2 Cyclic voltammograms in 0.1 M  $H_2SO_4$  containing 1.0 mM  $Cu^{2+}$ , 3.0 mM  $Fe^{3+}$ , or both (scan rate:  $0.10$  V  $s^{-1}$ ; electrode area:  $0.63$  cm<sup>2</sup>; MSE is mercurous sulphate reference electrode,  $+0.400$  V versus saturated calomel electrode). a, Au in  $Cu^{2+} + Fe^{3+}$ ; b, Au/TBEA ( $\sim 80\%$  coverage) in  $Fe^{3+}$ ; c, Au/(TBEA+NP) in  $Fe^{3+}$ ; d, Au/(TBEA+OM) in  $Fe^{3+}$ ; e, Au/(TBEA+NP) in  $Cu^{2+}$ ; f, Au/(TBEA+NP) in  $Cu^{2+} + Fe^{3+}$ ; g, Au/(TBEA+OM) in  $Cu^{2+} + Fe^{3+}$ ; h, Au/(TBEA+OM) in  $Cu^{2+}$ ; i, Au in 0.1 M  $H_2SO_4$  containing 0.4 M ethyl acetoacetate and either  $Cu^{2+}$  (solid line) or  $Fe^{3+}$  (broken line). The ligand coverage was estimated from the minimal amount of charge required to block Au/(TBEA) with naphthol polymer (towards  $Fe^{3+}$ ) compared to that required to block a bare electrode of the same geometric area. Surface coverages of ca. 80% with TBEA are typical. Essentially the same curves as c and d are obtained, respectively, for Au/NP and Au/OM in either  $Cu^{2+}$ ,  $Fe^{3+}$ , or  $Cu^{2+} + Fe^{3+}$ . For c, e, f, naphthol polymer (NP) was deposited in a stirred solution containing 1.0 mM 1-naphthol in 0.5 M  $H_2SO_4$  by passing a constant anodic current for  $2.0$   $\mu A$  cm<sup>-2</sup> for 4.5 min. This corresponds to about 10 layers of NP deposited at ligand-uncovered sites of the electrode, which results in a NP film thickness comparable with that of the ligand monolayer itself.





**Fig. 3** RA-FTIR spectra of: *a*,  $\text{Cu}^{2+}$ -TBEA complex in a  $\text{Au}/(\text{Cu}^{2+}\text{-TBEA}+\text{OM})$  electrode (specimen obtained by immersion of  $\text{Au}/(\text{TBEA}+\text{OM})$  in saturated aqueous copper acetate solution adjusted to pH 7.5 with sodium acetate, for 1.5 h at 40–45 °C); *b*, TBEA in the electrode of *a*, after electrochemical removal of the  $\text{Cu}^{2+}$  by cycling three times in 0.1 M  $\text{H}_2\text{SO}_4$  between +0.35 V and –0.75 V (v. MSE); *c*, TBEA in bulk liquid. The spectra in *a* and *b* were produced by a weighted subtraction of  $\text{Au}/\text{OM}$  spectrum from the spectra of the respective electrodes in *a* and *b*. The spectral features in the 3,000–2,800  $\text{cm}^{-1}$  region represent various C–H stretch modes<sup>19,23</sup>. The peaks at 1,744 and 1,716  $\text{cm}^{-1}$  are C=O stretch modes of the ester and ketone functions, respectively, of TBEA in the keto form (ref. 30, pp. 150 and 204). The features between 1,660 and 1,510  $\text{cm}^{-1}$  are characteristic of the  $\text{Cu}^{2+}$ -acetoacetate complex (ref. 30, pp. 165 and 209), the bands around 1,660  $\text{cm}^{-1}$  (*a*, *b*) and 1,653–1,630  $\text{cm}^{-1}$  (*c*) thus being assigned to the conjugated ester and double bond of TBEA in the enol form (ref. 30, pp. 165 and 209) (see Fig. 1). The spectra in *a* and *b* were taken with parallel polarized radiation in the reflection-absorption (RA) mode, at an angle of incidence of 75° and a resolution of 4  $\text{cm}^{-1}$  (ref. 19). The spectrum in *c* was taken in the transmission mode, at the same resolution, using a thin film of pure TBEA between two NaCl windows.

membranes was compared with that of gold electrodes coated with the same ligand, but sealed with a thin electrodeposited polymeric film (~10–15 Å thick) of 1-naphthol (NP), known to suppress electrochemical reactivity<sup>28</sup> (Fig. 2c). Figure 2e and f show, respectively, voltammetric curves of an  $\text{Au}/(\text{TBEA}+\text{NP})$  electrode in  $\text{Cu}^{2+}$  solution and in a mixed solution of  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ . Although the electrode is inert to  $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$  peaks are clearly observed. The voltammogram of the soluble  $\text{Fe}^{3+}$ -ethylacetoacetate complex in Fig. 2i indicates that the absence of  $\text{Fe}^{3+/2+}$  peaks in Fig. 2c and f is not due to a possible shift in the redox potentials of complexed  $\text{Fe}^{3+}$ .

The qualitative behaviour of a typical  $\text{Au}/(\text{TBEA}+\text{OM})$  electrode, shown in Fig. 2d and g for solutions containing  $\text{Fe}^{3+}$  or both  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  ions, respectively, is similar to that of  $\text{Au}/(\text{TBEA}+\text{NP})$  (Fig. 2c and f); however, a considerably decreased background, virtually coinciding with the baseline, in Fig. 2d and g, indicates a clear improvement of the barrier properties for the mixed monolayer membrane. Thus the selective complexation of  $\text{Cu}^{2+}$  enables its penetration into the monolayer and electron exchange with the underlying electrode, whereas hydrated  $\text{Fe}^{3+}$  remains in the bulk solution at considerably greater distance from the electrode, which precludes its

electrochemical reduction in the applied voltage range.  $\text{Fe}^{3+}$  forms a red complex with dissolved TBEA, possibly an oligomeric octahedral complex. For steric reasons, this should be prevented when the ligand is bound to the surface in an oriented monolayer. Total suppression of voltammetric response is also observed with other ions, which are either trivalent (for instance,  $\text{Ce}^{3+}$ ) or sterically incompatible (for instance,  $\text{VO}^{2+}$ ).

The monolayers on gold were characterized by contact-angle measurements<sup>19,22,24,26</sup>, reflection-absorption Fourier transform infrared (RA-FTIR) spectroscopy<sup>19,20,23</sup>, and various electrochemical measurements<sup>19,21</sup>, a full discussion of which is beyond the scope of this paper. Indirect electrochemical evidence indicates ~50% ligand coverage in the present (TBEA+OM) mixed monolayers. Surprisingly high contact angles for  $\text{Au}/(\text{TBEA}+\text{OM})$ , indicative of a rather unusual mode of film packing (typical values: 108°, 59° and 57° for water, bicyclohexyl and *n*-hexadecane, respectively, with no hysteresis) and significant contact angle variations are observed after  $\text{Cu}^{2+}$  uptake and removal. Figure 3 shows selected infrared spectra for the  $\text{Au}/(\text{TBEA}+\text{OM})$  system, from which a number of general conclusions can be drawn: (1) complexed TBEA (enol form) can be detected spectroscopically in the monolayer on gold; (2) the enol form is preserved upon electrochemical removal of the  $\text{Cu}^{2+}$ ; (3) differences in the relative intensities of the various C–H stretch peaks in Fig. 3a, b and c are indicative of non-random orientation of TBEA on the surface<sup>19,23</sup>; (4) the system appears stable towards electrochemical treatment.

Common prominent features in Fig. 2f and g, which may be correlated with structural characteristics of an ordered  $\text{Cu}^{2+}$ -selective monolayer barrier, are: (1) the complete absence of  $\text{Fe}^{3+/2+}$  peaks; (2) the absence of Cu UPD peaks; (3) the negative shift of the bulk Cu deposition peak by ~0.25 V; (4) the existence of a loop when the voltammetric scan direction is reversed. The absence of Cu UPD peaks indicates that Cu atoms are deposited inside the organized monolayer at some distance from the surface, not in direct contact with the gold substrate. The shift in the bulk Cu deposition potential and the voltammetric loop are indicative of the preferred perpendicular orientation of the ligand. In a monolayer where TBEA molecules are oriented normal to the substrate plane (as in Fig. 1), the complexed  $\text{Cu}^{2+}$  ions are held at a distance of ~7 Å from the Au surface, which introduces a tunnelling barrier for electron transfer with the underlying electrode (assuming that quantum-mechanical tunnelling governs electron transfer over distances of this order of magnitude). However, reduced Cu atoms are not complexed and may be deposited within the ligand 'cavity' closer to the electrode, thus lowering the energy barrier for further electron transfer. Voltammetrically, the initial barrier is manifested as an increased overpotential for the reduction, i.e. a negative shift of the reduction potential, whereas the easier electron transfer provided by the first layer of deposited Cu atoms gives rise to an autocatalytic effect, appearing as an enhanced reduction current (and thus a loop) on the reverse scan. Experiments with decreasing reversal potentials (not shown) indicate that even sub-monolayer amounts of Cu atoms may cause such appreciable catalytic effects.

From the negative shift of the  $\text{Cu}^{2+}$  reduction peak potential, observed with  $\text{Au}/(\text{TBEA}+\text{OM})$  relative to a bare gold electrode (Figs 2g and i), and considering a tunnelling mechanism for the electron exchange with the metal, one can estimate the potential shifts to be expected for varying distances between  $\text{Cu}^{2+}$  and the underlying electrode. To verify these expectations, a new ligand molecule which contains an additional methylene group, 3,3'-thiobispropyl acetoacetate,  $\text{S}(\text{CH}_2\text{CH}_2\text{CH}_2\text{OCOCH}_2\text{COCH}_3)_2$  (TBPA), was prepared and tested under identical electrochemical conditions. Assuming a similar orientation of the two ligands with respect to the substrate, the metal ion with TBPA should be located 0.5–1.0 Å further away from the gold surface than with TBEA. Indeed, this results in a reduction peak potential more negative by

$\sim 0.030$  V for the deposition of Cu with TBPA (Fig. 2g and h), which agrees qualitatively with the proposed tunnelling mechanism.

Thus we have demonstrated the possible use of spontaneous molecular organization at liquid-solid interfaces for the preparation of solid-supported functional monolayer structures. Structure-function interrelations in supramolecular organizes of this type appear to be advantageously revealed by electrochemical techniques. Such artificial self-assembled systems are of particular significance in view of their resemblance to the natural bilayer membranes (with respect to their molecular dimensions, mode of organization, and spontaneous formation), while offering high stability and the option of systematic modification.

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## Pb-Pb dating of young marbles from Taiwan

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Direct determination of the depositional age of a sedimentary sequence usually relies on isotopic analyses of authigenic minerals such as glauconite<sup>1</sup> and fine-grained clay minerals<sup>2-4</sup>. Moorbath *et al.*<sup>5</sup> recently reported the first direct dating of sedimentary carbonates using the whole-rock Pb-Pb method. The success of this method depends on the chemical fractionation of U from Pb and the consequent variation in <sup>206</sup>Pb/<sup>204</sup>Pb and <sup>207</sup>Pb/<sup>204</sup>Pb ratios. The high values and large variation of U/Pb ratios in sedimentary carbonates, long known to marine geochemists, have, however, not been exploited by geochronologists until recently<sup>5,6</sup>. Here I report a Pb-Pb isochron age for a relatively young carbonate sequence in Taiwan and demonstrate that the long-lived U-Pb decay scheme can be used to determine events as young as Mesozoic age. The new results also lead to the identification of an early Jurassic ( $\sim 170$  Myr BP) thermal event, which has never before been recognized in the polymetamorphic schist belt (the Tananao Schist) of Taiwan, but is widespread in southeastern continental China and other parts of eastern Asia<sup>7-10</sup>.

The island of Taiwan displays many important neotectonic features at the junction of two lithospheric plates: Eurasia and the Philippine Sea. The geological evolution of Taiwan involved a complex interplay of rifting, drifting, subduction polarity reversal and arc-continent collision<sup>11-16</sup>. Much of the island was assembled nearly *in situ* by deposition of miogeoclinal and/or continental slope strata. Exotic and suspect terranes and far-travelled ophiolite blocks were incorporated in this sector of the Asiatic margin by convergent or oblique underflow. These include: (1) Mesozoic amphibolite and serpentinite lenses in the Tailuko belt; (2) Mesozoic trench-argillite plus mélange complex of the Yuli belt; (3) Mio-Pliocene blueschist tectonic blocks enclosed in the Yuli belt; (4) the Neogene Luzon andesitic arc (the Coastal Range of Taiwan); and (5) the Miocene East Taiwan Ophiolite, emplaced as olistostromal debris in the Lichi Mélange of the Coastal Range.

The succession and timing of major tectonothermal events in Taiwan have been delineated using various isotopic systems

(Rb-Sr, U-Pb, Sm-Nd, K-Ar) in rocks and minerals of the Tananao Schist Complex<sup>17</sup>, and are summarized in Fig. 1. It was recognized that an important thermal episode associated with the early Yanshan Orogeny (150-180 Myr BP) in the southern part of mainland China (South China fold belts) was not detected in Taiwan by then available radiometry data<sup>17</sup>. Yet geological evidence suggests that a period of regional metamorphism, which produced the amphibolite-facies rocks now occurring as amphibolite lenses or enclaves, must have taken place before the emplacement of their host granitic magmas  $\sim 85$ -90 Myr ago<sup>17</sup>. We now show that this important thermal event was well recorded in the U-Pb systems of the recrystallized marble sequence of the Tananao Schist Complex.

The pre-Cenozoic Tananao Schist Complex is the oldest basement complex in Taiwan. It bears evidence of polymetamorphism and multiple deformation<sup>11,12,17-22</sup>. Lithologically, the complex can be divided into a western Tailuko belt, characterized by pelitic schists, granitic gneisses, marbles, intercalated mafic and tuffaceous metasediments, greenschists, amphibolites and minor lenses of serpentinites; and an eastern Yuli belt, characterized by a mélange of pelitic, argillitic and less voluminous mafic and ultramafic schists of oceanic affinities<sup>12,23-25</sup>. Carbonate strata and granitoids are conspicuously absent from this Yuli terrane. More detailed geological descriptions can be found elsewhere<sup>12,14</sup>.

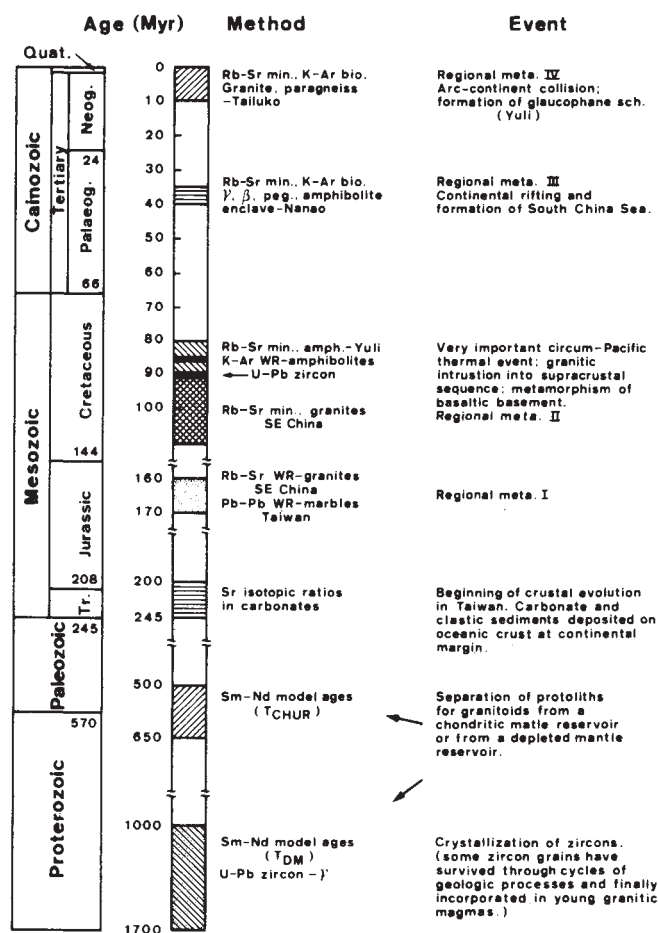
The Permo-Triassic limestones and interlayered clastics of the Tailuko belt are believed to be the oldest rocks exposed in Taiwan<sup>11,14,17,26</sup>. Using mineral parageneses, compositional variations and element partitioning between coexisting phases, these sedimentary strata are believed to have been subjected to a Mesozoic amphibolite-facies metamorphism with temperature  $T = 550$ -650 °C and fluid pressure  $P = 5$  kbar, followed by one or more periods of lower-grade metamorphic recrystallization in Cenozoic times<sup>11,12,23</sup>. The post-depositional prograde metamorphism of limestone into marble must have occurred before the granitic intrusions dated at  $\sim 85$ -90 Myr. In the present study, marble samples were collected from different outcrops from the northern part of the belt, all but one within 30 km of each other (Fig. 2). A previous Sr isotope study<sup>27</sup> and unpublished Sr isotope data reveal that the marbles are not homogeneous in Sr isotopic compositions, ranging from 0.7071 to 0.7080. They contain little or no Rb ( $<0.2$  p.p.m.) and quite variable Sr concentrations (90-3,470 p.p.m.). In all cases, radiogenic Sr growth is negligible. Using the recently proposed sea-



**Table 1** U and Pb concentrations and Pb isotope data for marbles from Taiwan

| Sample no.                  | U (p.p.m.) | Pb (p.p.m.) | $^{238}\text{U}/^{204}\text{Pb} (= \mu)$ | $^{206}\text{Pb}/^{204}\text{Pb}$ | $^{207}\text{Pb}/^{204}\text{Pb}$ | $^{208}\text{Pb}/^{204}\text{Pb}$ |
|-----------------------------|------------|-------------|------------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
| BJ82-121                    |            |             |                                          | 19.753                            | 15.728                            | 38.919                            |
| BJ82-122                    |            |             |                                          | 20.447                            | 15.688                            | 38.281                            |
| BJ82-126                    |            |             |                                          | 55.646                            | 17.424                            | 38.456                            |
| BJ82-127W                   |            |             |                                          | 112.315                           | 20.206                            | 38.622                            |
| BJ82-127D                   |            |             |                                          | 131.098                           | 21.169                            | 38.571                            |
| BJ82-147                    |            |             |                                          | 19.898                            | 15.667                            | 38.523                            |
| LBJ-1                       | 1.58       | 0.110       | 1,251                                    | 44.729                            | 16.877                            | 38.212                            |
| LBJ-2                       | 0.95       | 0.303       | 218                                      | 25.270                            | 15.894                            | 38.450                            |
| LBJ-3                       | 0.46       | 0.495       | 62                                       | 21.857                            | 15.734                            | 38.254                            |
| LBJ-4                       | 0.71       | 0.375       | 150                                      | 35.747                            | 16.507                            | 38.688                            |
| LBJ-5                       | 2.40       | 0.207       | 1,206                                    | 63.285                            | 17.775                            | 38.681                            |
| LBJ-6                       | 0.72       | 0.156       | 358                                      | 33.999                            | 16.321                            | 38.908                            |
| LBJ-7                       | 0.26       | 0.091       | 236                                      | 39.524                            | 16.619                            | 38.313                            |
| LBJ-8                       | 0.996      | 0.462       | 195                                      | 48.125                            | 17.131                            | 38.465                            |
| LBJ-9                       | 0.76       | 0.520       | 97                                       | 21.117                            | 15.801                            | 38.830                            |
| LBJ-10                      | 2.53       | 0.129       | 3,048                                    | 119.555                           | 20.656                            | 38.439                            |
| LBJ-11                      | 2.54       | 0.225       | 1,083                                    | 54.214                            | 17.363                            | 38.639                            |
| Average                     | 1.26       | 0.279       | 719                                      |                                   |                                   |                                   |
| NBS-981 (recommended)       |            |             |                                          | 16.934                            | 15.491                            | 36.721                            |
| NBS-981 (measured)          |            |             |                                          | $16.905 \pm 5$                    | $15.449 \pm 8$                    | $36.593 \pm 33$                   |
| Fractionation per unit mass |            |             |                                          | 0.1%                              | 0.09%                             | 0.09%                             |

Concentrations were determined by isotope dilution, using 97.844%  $^{235}\text{U}$  and 99.967%  $^{208}\text{Pb}$  spikes. Precision for all isotope ratios is better than 0.1%. All ratios have been corrected for isotopic fractionation by 0.1% per unit mass against the NBS-981 Pb standard. Pb blank  $\leq 2$  ng.



**Fig. 1** Summary of important tectonothermal events in Taiwan (modified from ref. 17). Symbols:  $\gamma$ , granite;  $\beta$ , basalt. The original 'missing' event of 160–170 Myr BP in Taiwan<sup>17</sup> is now established by the new Pb–Pb isochron data and is identified as regional metamorphism I.

water Sr isotopic evolution curves<sup>28–30</sup>, the available Sr isotope data suggest that the limestones were probably deposited in the Permian (230–270 Myr BP), consistent with the rare fossil record<sup>26</sup>.

Pb isotopic analyses were carried out on a Cameca TSN206 mass spectrometer. Pb samples were extracted from ~2 g of clean chips, using only HCl dissolution and ion-exchange techniques. Undissolved residues (graphite and silicates) were discarded because only Pb co-precipitated with carbonates was of prime interest. In addition, LBJ-subset samples were analysed for their U and Pb concentrations by the isotope dilution method. This was done in order to understand the general range of Pb contents and U/Pb ratios in the marbles (Table 1). Uncertainties for all Pb isotope ratios are 0.1% and the within-run precision ( $2\sigma_m$ ) was generally better than 0.07%. Line-fitting for isochron age computation was carried out following the procedures of York<sup>31</sup>. The quoted errors are at the 95% confidence level and were multiplied by the square root of the MSWD (mean square of weighted deviates) when MSWD itself was greater than unity.

The Pb isotope data are presented in Table 1 and displayed in an isochron diagram (Fig. 3). Owing to the very large variation in  $^{206}\text{Pb}/^{204}\text{Pb}$  ratios, the 17-point data set defines an isochron or errochron (MSWD = 6.6) age of  $166 \pm 33$  ( $2\sigma$ ) Myr, with a model  $\mu_1$  value of 8.34 (assuming the age of the Earth is 4.55 Gyr). This is probably the youngest Pb–Pb isochron age determined on any geological material. Note that the calculated  $^{207}\text{Pb}/^{206}\text{Pb}$  slope ( $0.049391 \pm 0.000703$ ) has a relatively small error (~1.4%), but for geologically young rocks, the small percentage slope error would introduce a rather large uncertainty in the calculated age (20% in this case). A similar percentage error in slope for ~2.84 Gyr-old Archaean carbonates<sup>5</sup> caused only ~1% uncertainty in the resulting isochron age. Nevertheless, the present Pb–Pb age of  $166 \pm 33$  Myr is regarded as the time of limestone recrystallization during the first period of regional metamorphism when some mid-ocean-ridge-type basalts<sup>12,23</sup> were also transformed to amphibolites. This age also falls in the period of the early Yanshan orogeny of southeastern China and supplies the 'missing' event to be found in Taiwan<sup>17</sup>.

The very large range of  $^{206}\text{Pb}/^{204}\text{Pb}$  ratios (~20–130) in the marble samples suggests that the *in situ*  $\mu$  ( $^{238}\text{U}/^{204}\text{Pb}$ ) values

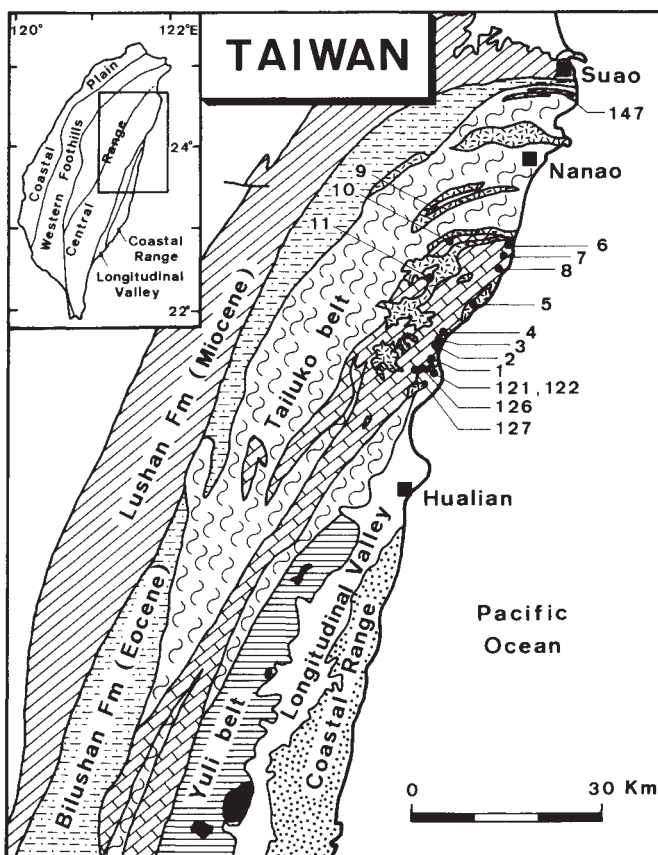


Fig. 2 Simplified geological map showing sampling localities (prefixes BJ82- and LBJ- for sample numbers are dropped for clarity).

would be extremely high, ranging from  $\sim 80$  to  $\sim 4,350$  since 166 Myr ago, assuming an initial  $^{206}\text{Pb}/^{204}\text{Pb}$  of 17.64 as calculated from a two-stage fit (see Fig. 3 legend). Such extraordinary U/Pb fractionation is more pronounced than that observed in the  $\sim 2.84$ -Gyr-old Archaean stromatolitic limestone of southern Zimbabwe, which gave *in situ*  $\mu$  values ranging from  $\sim 20$  to 113 (ref. 5). This inferred high  $\mu$  range for the marbles from Taiwan is confirmed by the U and Pb concentration determinations (Table 1) and is also supported by a  $\gamma$ -ray radiometric survey<sup>32</sup> in the marble areas of the Tananao Schist Complex. The  $\gamma$ -ray results show the U concentrations in nine U-enriched 'anomalous' marbles to be  $15 \pm 8$  (1 $\sigma$ ) and in 22 'normal' marbles to be  $\sim 1.4$  p.p.m.; whereas the Pb concentrations, as determined by another method, are uniformly less than 0.5 p.p.m. A rough estimate of U/Pb ratios yielded a range of  $\mu$  values from  $\sim 220$  to 4,800, which is in approximate agreement with that deduced from the isotopic data. The  $\mu$  values for the LBJ-subset samples range from 62 to 3,048 (Table 1).

The wide range of  $\mu$  values or U/Pb ratios and the mechanism of U/Pb fractionation in marble and limestone samples are not yet well understood. Sea water contains  $\sim 3 \text{ ng g}^{-1}$  of U and  $\sim 2 \text{ pg g}^{-1}$  of Pb (ref. 33), so that sea water has a U/Pb ratio of  $\sim 1,500$  or a  $\mu$  value of  $\sim 100,000$ . By contrast, mean river water has  $\sim 0.24 \text{ ng g}^{-1}$  of U and  $1 \text{ ng g}^{-1}$  of Pb (ref. 33), or a U/Pb ratio of  $\sim 0.24$ . Incorporation of U in carbonate rocks probably involves a series of processes, including reduction of soluble uranyl ( $\text{UO}_2^{2+}$ ) to insoluble uranous ( $\text{U}^{4+}$ ) ion, adsorption of U by organic or carbonaceous matter, and co-precipitation of organic matter and calcite to form sediments. Initial U/Pb ratios in limestones were likely to be high but not necessarily uniform. Diagenetic processing of carbonates involving continent-derived

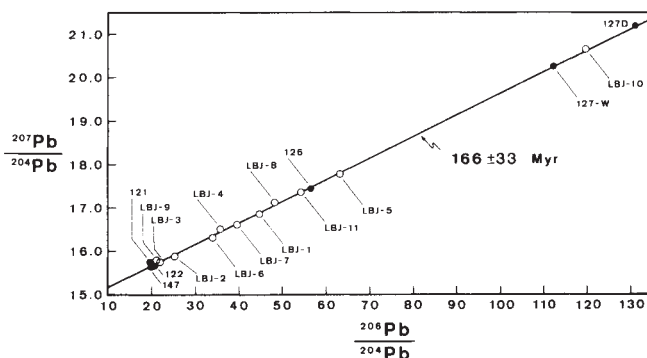


Fig. 3 Pb-Pb isochron diagram for the marbles from Taiwan. The 17-point data array (MSWD=6.6) yielded a slope of  $0.049391 \pm 0.000703$  ( $2\sigma$ ). Initial Pb isotopic compositions and  $\mu_1$  ( $^{238}\text{U}/^{204}\text{Pb}$ ) value were calculated using 4.55 Gyr for the age of the Earth and decay constants  $\lambda_{238} = 0.155125 \text{ Gyr}^{-1}$  and  $\lambda_{235} = 0.98485 \text{ Gyr}^{-1}$ , and assuming a two-stage evolution: ( $^{206}\text{Pb}/^{204}\text{Pb}$ )<sub>0</sub> = 17.64; ( $^{207}\text{Pb}/^{204}\text{Pb}$ )<sub>0</sub> = 15.56;  $\mu_1 = 8.34$ .

groundwaters as pore fluid may have caused partial reduction of the U/Pb ratio, depending on the water/rock ratio and water circulation paths. Later metamorphic recrystallization could have further modified U/Pb ratios and largely homogenized Pb isotopic compositions. The relatively small but reproducible scatter of data points in Fig. 3 suggests that Pb isotopic homogenization was not complete; this is particularly evident for rocks with low  $\mu$  values or low  $^{206}\text{Pb}/^{204}\text{Pb}$  ratios. As the samples were collected from a fairly large area (Fig. 2), such a degree of data scatter is probably not unexpected; even so, the scatter is no more than that obtained from small-scale sampling from a single drill core<sup>5</sup>. It is not clear whether the present data suggest a true regional Pb isotope homogenization or only homogenization within individual domains which happened to have similar average U/Pb ratios during the amphibolite-facies metamorphism  $\sim 170$  Myr ago. Nevertheless, the overall good linearity implies that the isochron age is geologically significant.

The present study thus shows that events as young as Mesozoic can be dated by the Pb/Pb technique. With this degree of U/Pb fractionation, the technique might even be applicable to dating Cenozoic carbonate strata. In the framework of a new tectonic evolution model of South China (K. J. Hsü *et al.*, in preparation), the newly found 170-Myr event corresponds well with the time when two microcontinental blocks, the Cathaysian and East-Sea, collided and amalgamated. This collisional event resulted in the widespread granitic intrusions and metamorphism of the Yanshan orogeny in southeastern China.

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## Chemical and isotopic evidence for lithospheric thinning beneath the Rio Grande rift

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Continental rifting disrupts the lithosphere, thermally and chemically modifying the upper mantle and crust beneath a rift. The isotopic and chemical compositions of basaltic rocks associated with continental rifting reflect the physiochemical conditions of their mantle source reservoirs and can be used as probes to trace processes associated with modification of the upper mantle. Basalts of different ages can potentially record the evolution of mantle reservoirs beneath rifts. Late Cenozoic alkali basalts from the Rio Grande rift and adjacent tectonic provinces display a wide range of isotopic values that correlate with tectonic setting and upper-mantle geophysical properties. This correlation indicates that the upper mantle beneath the rift region is composed of two distinct geochemical reservoirs<sup>1</sup> that, before lithospheric extension, corresponded to the ancient lithospheric mantle and asthenosphere, part of the convecting upper mantle. Here we discuss major-element and isotopic data for basalts in a small region of the southeastern Colorado Plateau transition zone adjacent to the central Rio Grande rift. Basalts from this area record an episode of lithospheric thinning that occurred between 8 and 4 Myr ago, concurrent with regional uplift and reactivation of rifting.

The Rio Grande rift<sup>2-5</sup> was formed during the extensional deformation that affected much of the western United States during the middle and late Tertiary<sup>6</sup>. It is unique among continental rifts in that it is part of a broad (>1,200 km) region of extended lithosphere and anomalous upper mantle that includes the Basin and Range province and the margins ('transition zone'<sup>7</sup>) of the Colorado Plateau. The northern rift consists of a well-defined series of asymmetrical grabens situated between the Colorado Plateau and the Great Plains (Fig. 1). The southern rift, where the earliest and most extension has occurred<sup>8</sup>, is physiographically indistinguishable from the adjacent Basin and Range province.

Late Cenozoic alkali basaltic rocks from the Rio Grande rift and adjacent tectonic provinces of New Mexico and Arizona have a wide range of Nd isotopic ratios that correspond to  $\epsilon_{Nd}$  values of +8.3 to -0.6<sup>1,9-16</sup>. Alkali basalts with  $\epsilon_{Nd}$  values throughout this range carry mantle-derived peridotite xenoliths, indicating that the magmas ascended rapidly through the crust with little opportunity for crustal interaction to alter their isotopic compositions from those that represent the mantle source reservoirs. The Nd isotopic compositions of alkali basalts correlate with location, and more importantly, with tectonic setting (Figs 1 and 2). Alkali basalts from the southern Basin and Range province and the southern Rio Grande rift have the highest  $\epsilon_{Nd}$  values. Alkali basalts and nephelinites from the Great Plains, Colorado Plateau and northern Rio Grande rift have the lowest  $\epsilon_{Nd}$  values. Alkali basalts with intermediate  $\epsilon_{Nd}$  values occur within the southeastern Colorado Plateau transition zone.

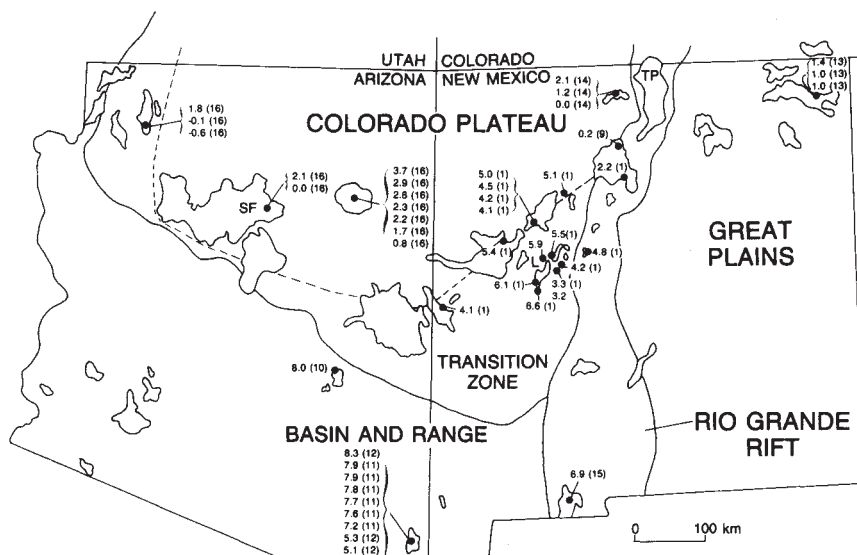
The correlation of isotopic composition with tectonic setting is a major constraint in interpreting mantle source regions. It suggests that two distinct mantle reservoirs exist beneath the rift and adjacent tectonic provinces (Fig. 2). In general these two geochemical reservoirs are identifiable with the lithospheric mantle (low  $\epsilon_{Nd}$  values) and asthenospheric mantle (high  $\epsilon_{Nd}$  values), as defined by seismic wave velocities and densities. However, as discussed below, during active rifting there are periods when the geochemical and geophysical reservoir boundaries do not correspond. In recognition of this discrepancy, Perry *et al.*<sup>1</sup> referred to these mantle reservoirs as 'depleted' mantle (DM), producing basalts with  $\epsilon_{Nd} = +7$  to +8, and 'enriched' mantle (EM), producing basalts with  $\epsilon_{Nd}$  values of 0 to +2.

The proposed correspondence of EM to lithospheric mantle and of DM to asthenosphere before regional extension is based on the correlation of isotopic compositions with upper-mantle geophysical properties in regions of contrasting tectonic history<sup>1</sup>. For instance, the Great Plains (Fig. 1) is part of the continental craton that has been relatively unaffected by Cenozoic tectonics. It is underlain by thick subcrustal lithosphere (~100 km) with a density of 3.32 g cm<sup>-3</sup> and normal upper-mantle  $P_n$  velocities of 8.2 km s<sup>-1</sup> (refs 17, 18). Mantle-xenolith-bearing nephelinites and basanites from the Great Plains have  $\epsilon_{Nd} = +1.0$  to +1.4<sup>13</sup>, values we assign to EM. The occurrence of EM-derived volcanic rocks in this region of the craton indicates that EM corresponds to lithospheric mantle in regions where there has been no extension.

In contrast to the Great Plains, the lithosphere of the southern Basin and Range province and the southern Rio Grande rift has undergone the greatest amount of extension in the rift region. Alkali basalts from these areas contain mantle-derived xenoliths and have  $\epsilon_{Nd}$  values clustering at +7 to +8<sup>10-12,15</sup>, values we assign to DM. As asthenospheric mantle, marked by anomalously low  $P_n$  velocities (7.7-7.8 km s<sup>-1</sup>) and density (3.17 g cm<sup>-3</sup>) extends upward to the base of the crust (~30 km) beneath both the southern Basin and Range and the southern Rio Grande rift<sup>18-21</sup>, the DM isotopic signatures probably characterize asthenosphere that has completely replaced lithospheric mantle.

Within the Rio Grande rift region there is a gradual change from south to north in the degree to which the geochemically defined and geophysically defined lithospheric mantle correspond. This discrepancy is largest in the north, where the amount of lithospheric extension is least. Beneath the axial grabens of the northern rift,  $\epsilon_{Nd}$  values (+0.2 to +2.2) of alkali basalts indicate that these basalts were derived from EM and that relatively little replacement of EM by DM has occurred. Yet low  $P_n$  velocities (~7.7 km s<sup>-1</sup> (refs 22, 23)) and low mantle densities (3.22 g cm<sup>-3</sup>), modelled from gravity data<sup>24</sup>, indicate that asthenosphere here extends upward to the base of the crust (30-35 km). Thus EM does not correspond with lithospheric mantle beneath this region. We suggest that beneath the northern

**Fig. 1** Regional variation of  $\epsilon_{\text{Nd}}$  for uncontaminated late Cenozoic alkali basalts, basanites and nephelinites of the Rio Grande rift and adjacent tectonic regions. Sources of data are given next to the  $\epsilon_{\text{Nd}}$  values.  $\epsilon_{\text{Nd}} = 10^4[(^{143}\text{Nd}/^{144}\text{Nd})_{\text{rock}} / (^{143}\text{Nd}/^{144}\text{Nd})_{\text{chondrite}} - 1]$ . Dashed line is the boundary of the Colorado Plateau interior, modified from ref. 7. Sample localities in volcanic fields for which multiple samples exist or for which exact sample localities are not known are generalized to a single point, except for in the Lucero volcanic area (L).  $\epsilon_{\text{Nd}}$  for contaminated alkali basalts<sup>1</sup> of the Taos Plateau (TP), which range from -1 to -4 (ref. 14), are not shown.  $\epsilon_{\text{Nd}}$  for feldspathic basalts of the San Francisco volcanic field (SF) range from +0.2 to -2.6 (ref. 16) and are not shown because they form a distinct petrologic and isotopic group compatible with an origin involving contamination by low  $^{87}\text{Sr}/^{86}\text{Sr}$  lower crustal wall rock that is inferred to exist beneath much of this region<sup>1</sup>.



rift EM-type lithospheric mantle has been 'thermally thinned' and converted to asthenosphere, while retaining its geochemical properties.

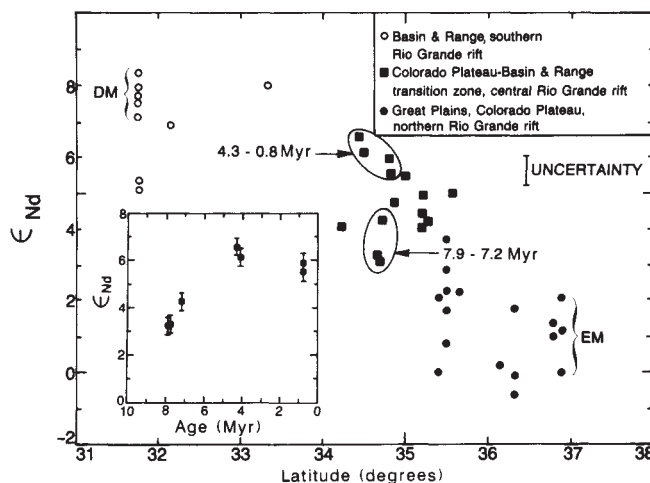
An intermediate situation is indicated for the transition zone of the Colorado Plateau. Here the alkali basalts have intermediate  $\epsilon_{\text{Nd}}$  values, indicating that their source contained both DM and EM components. The most likely place for both components to be consistently involved in alkali basalt genesis is at the DM/EM boundary, indicating that the DM/EM boundary beneath the transition zone is shallower than beneath the northern rift. However, here, too, anomalously low  $P_n$  velocities indicate that the asthenosphere is nearly in contact with the base of the crust, suggesting that the lithospheric mantle beneath the transition zone has been thermally converted to asthenosphere and partly replaced by DM.

We conclude from these observations that rifting involves a change in the physical properties of the lithospheric mantle (heating) and a physical replacement of one type of chemical reservoir (EM) with another (DM). The former precedes the latter by a period of ~10 Myr. But the data cited are from different localities, each at a different stage of reservoir evolution; the evolution of reservoirs with time at any one locality is only inferred. If the implied erosion of EM and replacement of EM by DM is in fact occurring, it should be recorded in the isotopic compositions of basalts for a given small area, assuming (1) there is a long enough record of volcanism and (2) the rising DM/EM boundary moves through the equilibration depth of basalts during some portion of the volcanic record. In search of this confirming evidence we have conducted detailed field and geochemical studies of an area in the transition zone (the Lucero volcanic area; Fig. 1) where a relatively long record of basaltic activity is preserved (from 8.3 Myr ago to the present). In this area volcanism occurred in three intervals: 8.3-6.2, 4.3-3.3 and 1.1-0.3 Myr ago<sup>25</sup>.

The isotopic and major element compositions of basalts erupted in the Lucero area changed with time in the expected manner (Figs 2 and 3). Alkali basalts from the oldest volcanic interval have the lowest  $\epsilon_{\text{Nd}}$  values of +3.2 to +4.2, indicating derivation from a mixed DM-EM source dominated by EM. In the intermediate and youngest intervals, both alkali basalts and olivine tholeiites are erupted. Alkali basalts from the intermediate and youngest intervals have  $\epsilon_{\text{Nd}} = +6.1$  to +6.6, and +5.5 to +5.9, respectively, indicating a source region containing a significantly larger component of the underlying DM reservoir. As there is no systematic correlation between degree of silica undersaturation and isotopic composition among the samples from

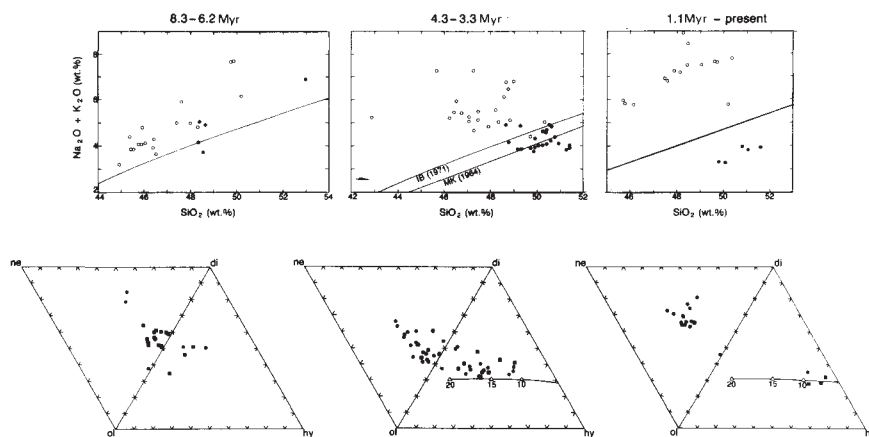
different age intervals, we do not think the differences in  $\epsilon_{\text{Nd}}$  reflect systematic differences in the depth of equilibration. Instead, we think that the larger DM component in the younger basalts reflects erosion of EM and the rise of the DM/EM boundary between 8 and 4 Myr ago (Fig. 4). The fact that  $\epsilon_{\text{Nd}}$  values of the youngest two intervals are essentially the same within error (Fig. 2, inset) has two likely explanations: either there was no rise of the DM/EM boundary in the period after 4 Myr ago in this area, or the basalts of the youngest cycle, which are situated 30 km north of basalts of the intermediate interval (Fig. 1), record the rise of a DM/EM boundary that is lagging slightly behind the rise of the DM/EM boundary farther south in the transition zone.

Olivine tholeiites comprise >80% of the volume of volcanic rocks in the Lucero area and were erupted only during the youngest two intervals. On the basis of their isotopic compositions and on experimental evidence, Perry *et al.*<sup>1</sup> concluded that



**Fig. 2** Variation of  $\epsilon_{\text{Nd}}$  with latitude for uncontaminated alkali basalts of the Rio Grande rift region. Data sources are the same as in Fig. 1. Ranges of EM and DM are based on data cited from ref. 1, which did not include more recent data from the southern and southwestern Colorado Plateau<sup>16</sup>. Alkali basalts from the Lucero volcanic area (circled samples and inset) record the change in  $\epsilon_{\text{Nd}}$  of alkali basalts within a small area of the Colorado Plateau transition zone between ~8 and 4 Myr ago.





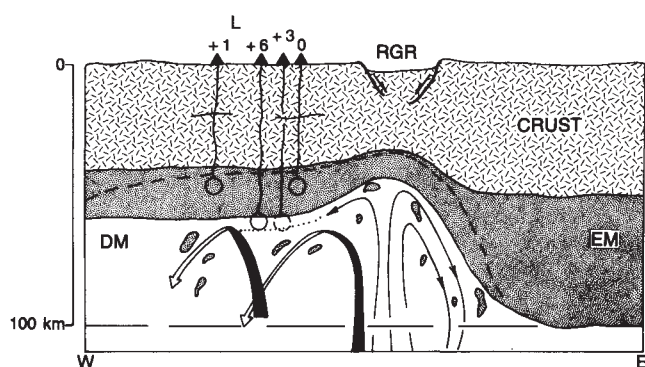
**Fig. 3** Compositions of basalts from the Lucero volcanic area. Upper: alkalis against silica. Open circles are nepheline-normative, solid circles are hypersthene-normative compositions. Lines labelled IB (1971) and MK (1964) divide alkali olivine basalts (above) from tholeiites, after refs 29 and 30 respectively. Lower: cation normative compositions. Compositions of basalts with  $Mg/(Mg + Fe^{2+}) > 0.60$ , where  $Fe^{3+}/(Fe^{2+} + Fe^{3+}) = 0.15$ , are shown by squares. Line labelled 10, 15, 20 shows loci of liquid in equilibrium with two pyroxenes and olivine of peridotite assemblage at indicated pressure (in kbar), from ref. 27.

these tholeiites equilibrated at shallower depths than the alkali basalts, entirely within EM. The evolution to large degrees of melting at shallower depths beginning at  $\sim 4$  Myr ago probably could not have occurred until the 'cold' EM/lithosphere was thermally converted to EM/asthenosphere beneath this area. The onset of tholeiitic volcanism in this area thus marks the thermal thinning of the uppermost mantle. Regional uplift, concurrent with reactivation of rifting and formation of the modern narrow rift basins, occurred in the rift region  $< 10$  Myr ago<sup>5</sup>, possibly within the interval 7–4 Myr ago<sup>6,8</sup>. Thermal expansion of the uppermost mantle accompanying conversion of lithospheric mantle to asthenosphere is a reasonable mechanism to explain at least a portion of the uplift of this region<sup>26</sup>.

We conclude as follows. (1) The shift in compositions records a significant shallowing of the zone of magma genesis, since

melt produced from peridotitic mantle is more hypersthene-normative at lower pressures<sup>27</sup>. The evolution of melting to shallower depths in the mantle followed thermal conversion of EM/lithosphere to EM/asthenosphere between 8 and 4 Myr ago. (2) The higher  $\epsilon_{Nd}$  values of alkali basalts of the intermediate and youngest intervals record a rise of the DM/EM boundary between 8 and 4 Myr ago. Thermal conversion of EM/lithosphere to EM/asthenosphere is followed by the erosion of EM and replacement by DM. We estimate the time lag between thermal conversion of lithospheric mantle and physical replacement of lithospheric mantle to be  $\sim 10$  Myr. (3) The thinning of lithospheric mantle correlates generally with regional uplift and reactivation of rifting and formation of the modern grabens of the rift<sup>8,28</sup>.

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**Fig. 4** Schematic E-W cross section through the central Rio Grande rift showing present configuration of EM and DM, depth of magma equilibration, and  $\epsilon_{Nd}$  of basalts. Long dashed line indicates the position of the DM/EM boundary before lithospheric extension, when DM corresponded to asthenosphere and EM corresponded to lithosphere. Short dashed line indicates the position of the asthenosphere/lithosphere boundary based on  $P_n$  velocities and gravity modelling (see text). Dotted line indicates probable position of the DM/EM boundary beneath the Lucero volcanic area (L) during the earliest volcanic event 8.3–6.2 Myr ago. Alkali basalts erupted during that event equilibrated (dashed circle) mainly within EM. In contrast basalts of the youngest events equilibrated mainly within DM owing to erosion of EM between 8–4 Myr ago. Tholeiites of the youngest events equilibrated at shallower depths in the mantle, entirely within EM that had been thermally converted to asthenosphere. Erosion of EM may proceed by convectively mixing the lower portions of thermally converted EM into the underlying DM/asthenosphere (indicated by arrows). Small-scale convection can be induced when hotter, upwelling asthenosphere is juxtaposed against cooler lithosphere beneath regions of extensionally thinned lithosphere<sup>31</sup>.

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# Preparation of hydrogen and lithium feldspars by ion exchange

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Alkali feldspars are among the most abundant rock-forming minerals. They are framework aluminosilicates with a basic stoichiometry of  $\text{MAlSi}_3\text{O}_8$ . The feldspar framework accommodates metal (M) ions ranging from about 1 to 1.5 Å ( $\text{Na}^+$  to  $\text{Rb}^+$ ) in size. Cations outside this range, such as lithium ions, have been found to enter the M site only in trace amounts, both in natural and synthetic samples. Hydrogen is also a common trace element in feldspars, but its presence seems to have considerable bearing on kinetic phenomena like oxygen diffusion and Al/Si order-disorder processes<sup>1</sup>. Neither these effects nor the structural roles of Li or H are well understood. It therefore seemed desirable to obtain the fully substituted feldspars  $\text{HAlSi}_3\text{O}_8$  and  $\text{LiAlSi}_3\text{O}_8$  for further study. In this letter I report on the low-temperature ion-exchange synthesis and lattice constants of these feldspars.

Direct synthesis of lithium or hydrogen feldspars seems impossible because other phases are more stable. Monovalent cations of the appropriate size range can be exchanged between feldspars and molten salts at ~800 °C without alteration of the framework<sup>2</sup>, but with Li salts other phases form, such as  $\beta$ - $\text{LiAlSi}_2\text{O}_6$ . Under hydrothermal conditions dissolution and reconstruction to new phases or phase assemblages is the prevailing mechanism.

Synthesis via ion exchange therefore requires that the exchange medium has no effective solubility for the feldspar and that the temperature of the exchange reaction is sufficiently low to suppress disintegration of the feldspar framework. With Li-Al framework silicates of the keatite and  $\beta$ -quartz type it has been shown<sup>3,4</sup> that these conditions can be met for Li-H exchange by using concentrated mineral acids for example  $\text{H}_2\text{SO}_4$ , at temperatures near their boiling points, as the exchange medium. The exchange then follows reactions such as



In this study the same approach has been applied to feldspars. A natural potassium feldspar from Volkesfeld, Eifel, West Germany, was used as the starting material. This feldspar was selected because it is very homogeneous, of gem-like quality, and because it has been studied in much detail before<sup>5-7</sup>. The composition in terms of its major components is ~84.5%  $\text{KAlSi}_3\text{O}_8$ , 14.2%  $\text{NaAlSi}_3\text{O}_8$  and 1.3%  $\text{BaAl}_2\text{Si}_2\text{O}_8$  (refs 5, 7). Structurally it is a member of the monoclinic alkali feldspars with a partially ordered Al/Si distribution (low sanidine)<sup>5</sup>. For ion-exchange experiments a clear, homogenous sample was ground to a size of <200 µm.

Ion-exchange experiments were carried out in  $\text{H}_2\text{SO}_4$  (98%) at 320 °C in a quasi-closed system (reflux condenser). Runs with the original sanidine for times of up to 72 h failed to produce any changes in the feldspar. This was ascribed to the small mobility of potassium, the predominant alkali cation in the natural sample, as compared with sodium<sup>1</sup>. Therefore, prior to

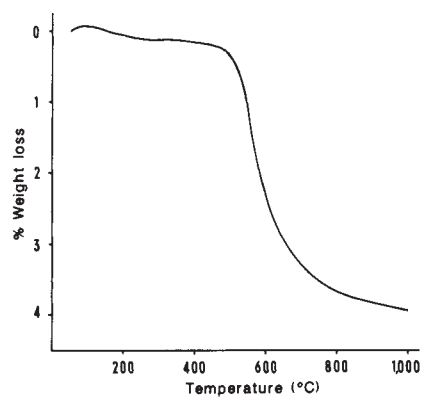


Fig. 1 Thermogravimetric analysis of H feldspar. Heating rate  $40 \text{ K h}^{-1}$ .

further acid treatment, the potassium content was exchanged against sodium ions in molten  $\text{NaCl}$  at 800 °C yielding the high albite equivalent of the original sanidine. Repeated exchange (four 24 h runs in  $\text{NaCl}$  of ~20 times the weight of feldspar) brought the residual  $\text{KAlSi}_3\text{O}_8$  content to less than 1% with the  $\text{BaAl}_2\text{Si}_2\text{O}_8$  content remaining unaffected (X-ray fluorescence results).

This high albite sample was then treated with  $\text{H}_2\text{SO}_4$ . After 24 h at 320 °C the Na content had fallen to ~5% of its original value. After an additional 24-h run it was <1%, the Ba, Al and Si contents remaining the same (XRF). A feldspar of composition  $\text{HAlSi}_3\text{O}_8$ , with only minor residual contents of the Na, K and Ba components, would have an  $\text{H}_2\text{O}$  content of 3.75 wt%. On then heating in air for 10 h at 600 °C plus 5 h at 650 °C a weight loss of 3.63% was observed.

The weight change with temperature is shown as a thermogravimetric curve in Fig. 1. For the heating rate applied ( $40 \text{ K h}^{-1}$ ) dehydration starts at ~500 °C and the final weight is reached at ~800 °C.

Infrared spectroscopy (feldspar powder in KBr pellets) also confirmed the presence of some form of water in the  $\text{H}_2\text{SO}_4$ -treated sample: broad absorption bands at ~3,100 and  $2,500 \text{ cm}^{-1}$  were found that were absent both in the high albite starting material and in the thermally dehydrated form. These bands therefore are assumed to be caused by OH stretching vibrations shifted by hydrogen bonding.

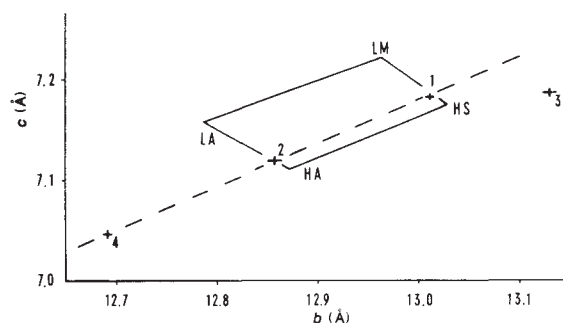
During dehydration the crystallinity of hydrogen-feldspar is progressively lost, as indicated by increasing X-ray line broadening. When the full weight loss is reached only the strongest reflections remain as very broad peaks. This is in contrast to the thermal behaviour of the hydrogen forms of other framework silicates<sup>4</sup> that can be dehydrated without loss of long range order.

The H feldspar can easily be re-exchanged to the Na form. Immersion in molten  $\text{NaNO}_3$  at 320 °C for 24 h, fully regenerates the starting high albite both chemically (XRF) and structurally (XRD). In the same way, by replacing  $\text{NaNO}_3$  with  $\text{LiNO}_3$ , complete Li-substitution can be achieved. This Li feldspar, by immersion in  $\text{NaNO}_3$ , can also be re-exchanged to the original albite. This process seems to be rather sluggish, as three consecutive runs at 320 °C for 48 h were needed before a product was

Table 1 Space groups and lattice constants of the Volkesfeld sanidine and its Na, H and Li forms

|                         | SG   | a (Å)     | b (Å)      | c (Å)     | $\alpha$  | $\beta$    | $\gamma$  | V (Å <sup>3</sup> ) |
|-------------------------|------|-----------|------------|-----------|-----------|------------|-----------|---------------------|
| (1) Sanidine, untreated | C2/m | 8.544 (4) | 13.010 (6) | 7.184 (4) | 90        | 115.99(4)  | 90        | 717.7(7)            |
| (2) Na exchange from 1  | C1   | 8.167 (8) | 12.856 (9) | 7.120 (4) | 93.34 (6) | 116.40 (6) | 90.22 (8) | 668.2 (10)          |
| (3) H exchange from 2   | C2/m | 7.946 (5) | 13.131 (7) | 7.189 (4) | 90        | 116.57 (6) | 90        | 671.0 (9)           |
| (4) Li exchange from 3  | C1   | 7.878 (2) | 12.690 (5) | 7.046 (3) | 95.89 (4) | 116.78 (3) | 90.13 (3) | 624.6 (5)           |





**Fig. 2** Plot of the *b*, *c*-lattice constants of alkali feldspars. LA and HA are the Na feldspars with fully ordered (respectively, fully disordered) Al/Si distribution, LM and HS are the respective K feldspars. For symbols 1–4 see Table 1. The broken line from 1 to 2 connects the K and Na feldspars of identical Al/Si order. The Li feldspar fits the extension of this line quite well, the H feldspar does not, indicating a different structural role for H as compared with the alkali ions.

obtained that according to XRF and XRD was identical to the starting high albite.

The treatments described above did not significantly alter the microscopical appearance of the feldspars. The individual grains often developed cracks, mostly following cleavage planes, but otherwise remained clear and intact.

The powder-X-ray diffractograms can be indexed without major problems. Least squares analysis using only well-resolved lines (11–15 reflections) yields the unit-cell data given in Table 1. The powder diffraction patterns are consistent with C2/m symmetry in the H form like that in the original sanidine and with  $C\bar{1}$  symmetry in the Na and Li forms. The unit cell volume of the H form is similar to that of the Na feldspar, whereas the Li feldspar has a much smaller cell volume. The distortions brought about by the Li ions apparently follow the trends seen in the other alkali feldspars. Figure 1 shows the alkali feldspar *b*–*c* plot<sup>8</sup>. Variation in the size and concentration of the alkali ions at constant framework Al/Si order shifts the lattice constants along the long sides of the parallelogram limited by the fully ordered and fully disordered Na and K feldspars. The Li feldspar fits quite well on the extension of the straight line connecting its Na and K equivalents; the H feldspar falls off considerably. The Li feldspar also plots on the extension of the sanidine-high albite line in an  $\alpha^*$ – $\gamma^*$  diagram. So, whereas Li distorts the feldspar framework, essentially in the way that would be expected for a very small cation, hydrogen causes a different type of distortion. In 'normal' alkali feldspars both the *a*- and *b*-lattice constants reflect the size of the cations, the Li feldspar having the smallest values reported so far, and also the smallest cell volume. The striking feature of the H feldspar is the length of its *b*-axis which exceeds that of all alkali feldspars, bringing its cell volume close to that of Na feldspars. Lattice constant variations in the alkali feldspars are essentially determined by the way the alkali ions 'fill' the cavity around the M position. The role of hydrogen certainly cannot be described in these terms because it can only bond to one or two oxygens. It is therefore conceivable that the framework geometry is affected by hydrogen only in particular directions whereas others, such as the *b* direction, are left free to expand without constraints by M-bonding. Single crystal studies now in progress will hopefully provide some understanding of the structural role of hydrogen in this feldspar.

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## Global trends in the nature of organic matter in river suspensions

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Riverine organic matter consists of a labile (metabolizable) and a residual (non-metabolizable) fraction<sup>1</sup>. The labile fraction can be oxidized or 'lost' within the rivers<sup>2</sup>, their estuaries<sup>3</sup>, and in the marine environment<sup>4</sup>. Previous estimates of the river fluxes of organic carbon have not considered such potential losses, being based on measurements of the bulk carbon and nitrogen contents<sup>5</sup>. Here I report detailed chemical analyses of organic matter associated with suspended matter from several major world rivers that have allowed me to differentiate it into labile and refractory fractions. Globally, 35% ( $81 \times 10^{12}$  g C yr<sup>-1</sup>) of it belongs to the labile fraction and may become oxidized in estuaries and in the marine environment. The rest ( $150 \times 10^{12}$  g C yr<sup>-1</sup>) appears to be highly degraded, with the bulk entering present-day tropical and subtropical sea areas. This degraded fraction could represent a significant source of organic carbon accumulating in marine sediments.

Samples were collected during the SCOPE/UNEP (Scientific Committee on Problems of the Environment/United Nations Environment Programme) study on the 'transport of carbon and minerals in major world rivers'<sup>6–8</sup>. The rivers were sampled at intervals of 4–6 weeks at locations near the mouth, where the river water is above the influence of sea water. Suspended matter collected by filtering the water samples through glass fibre filters (Schleicher & Schüll 6 vg 500 °C) were analysed for organic carbon (Carlo Erba), and after acid hydrolysis, for sugars and amino acids (Biotronik, Frankfurt) according to methods described elsewhere<sup>9</sup>. The hydrolysis conditions were 2 M HCl, 100 °C and 3.5 h for sugars, and 6 M HCl, 110 °C and 22 h for amino acids.

Sugars and amino acids were chosen for the study because of: (1) their quantitative significance in terms of the initial organic input into the aquatic sedimentary environment<sup>10</sup>; and (2) their labile nature, which makes them more susceptible to biodegradation<sup>11</sup>. Their relative contributions to the bulk particulate organic carbon (POC) are useful in quantifying the organic matter that may be lost from river suspensions. Conversely, they permit the estimation of the potential contribution of land-derived organic matter to the global annual organic carbon burial in marine sediments. Here, that fraction of the bulk POC contributed by sugars and amino acids will be termed labile particulate organic carbon (LPOC) and the remainder residual particulate organic carbon (RPOC).

For purposes of discussion and interpretation of results, I use a classification proposed by Meybeck<sup>12</sup>. The samples were classified into groups, each group representing samples within a specific range of the measured suspended-matter concentrations (0–15 mg l<sup>-1</sup>, 15–50 mg l<sup>-1</sup>, and so on). The averages of the various parameters such as total suspended solids (TSS), POC and LPOC, and their fluxes in each group were calculated. They were later used for the calculation of the global flux rates. (Fig. 1; Table 1).

**Table 1** Distribution of POC in river suspensions in the various concentration ranges

| TSS <sub>r</sub><br>(mg l <sup>-1</sup> ) | Q<br>(km <sup>3</sup> yr <sup>-1</sup> ) | TSS <sub>c</sub><br>(mg l <sup>-1</sup> ) | TSS <sub>i</sub><br>(Tg yr <sup>-1</sup> ) | TSS <sub>i</sub><br>(%) | POC <sub>c</sub><br>(%) | POC <sub>i</sub><br>(Tg yr <sup>-1</sup> ) | POC <sub>i</sub><br>(%) | LPOC<br>(%) |
|-------------------------------------------|------------------------------------------|-------------------------------------------|--------------------------------------------|-------------------------|-------------------------|--------------------------------------------|-------------------------|-------------|
| 0-15                                      | 1,420                                    | 8                                         | 11                                         | 0.06                    | 8.4                     | 0.9                                        | 0.4                     | 35.2        |
| 15-50                                     | 6,800                                    | 30                                        | 200                                        | 1.1                     | 3.6                     | 7.2                                        | 3.1                     | 46.6        |
| 50-150                                    | 11,970                                   | 80                                        | 960                                        | 5.0                     | 2.2                     | 21.1                                       | 9.1                     | 22.1        |
| 150-500                                   | 7,550                                    | 290                                       | 2,190                                      | 11.6                    | 1.3                     | 28.5                                       | 12.3                    | 11.8        |
| 500-1,500                                 | 8,750                                    | 975                                       | 8,530                                      | 45.0                    | 1.6                     | 136.0                                      | 58.9                    | 18.8        |
| 1,500-5,000                               | 670                                      | 3,000                                     | 2,010                                      | 10.6                    | 0.6                     | 12.1                                       | 5.2                     | 4.3         |
| 5,000-15,000*                             | 26                                       | 8,000                                     | 208                                        | 1.1                     | 0.5                     | 1.0                                        | 0.4                     | —           |
| 15,000-50,000*                            | 56                                       | 30,000                                    | 1,680                                      | 8.9                     | 0.5                     | 8.4                                        | 3.6                     | —           |
| >50,000*                                  | 48                                       | 65,000                                    | 3,120                                      | 16.5                    | 0.5                     | 15.6                                       | 6.8                     | —           |
| Total                                     | 37,290                                   |                                           | 18,909                                     |                         |                         | 231.0                                      |                         |             |

TSS, total suspended solids; Q, water discharge in each range; TSS<sub>c</sub>, average TSS concentrations in each range; TSS<sub>i</sub>, quantity of TSS transported in each range in 10<sup>12</sup> g yr<sup>-1</sup> (Tg yr<sup>-1</sup>) and as a percentage of global annual flux calculated by multiplying the TSS<sub>c</sub> by the total water discharge in the same ranges<sup>5</sup>; POC<sub>c</sub>, average particulate organic carbon content as a percentage of TSS; POC<sub>i</sub>, quantity of particulate organic carbon transported in Tg yr<sup>-1</sup> and as a percentage of global annual flux; LPOC, labile particulate organic carbon in % (calculated as the carbon contributed by sugars and amino acids to total POC).

\* Data from ref. 5.

POC contents are in the range 0.5–12% of TSS. They decrease with logarithmically increasing TSS concentrations, a trend similar to that reported previously<sup>5</sup>. This results from: (1) the dilution of autochthonous (riverine) organic matter by mineral matter (increased erosion)<sup>5</sup>; (2) reduced autochthonous input due to lack of light penetration at high sediment concentrations (turbidity)<sup>13</sup>; and (3) differences in the sources and biogeochemical processes affecting the nature of organic matter at various stages of the hydrographic regime<sup>14,15</sup>.

The distribution of POC fluxes within the various groups show that, of the total of 231 × 10<sup>12</sup> g C yr<sup>-1</sup> POC transported by the rivers, 59% occurs within the TSS concentration range 500–1,500 mg l<sup>-1</sup>, which accounts for 45% of the total sediment input into the sea (Table 1). Normally such high TSS concentrations are observed in rivers draining into tropical and subtropical sea areas of Asia and the Far East<sup>14,15</sup>.

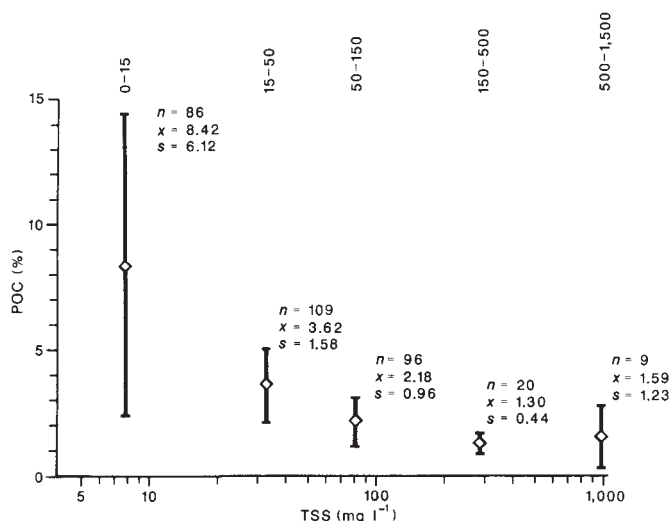
The LPOC distribution pattern in suspended matter from the individual rivers shows considerable regional variations (Fig. 2). In rivers such as the Mackenzie, St Lawrence and the Parana it contributes 35–40% to the total POC. For the Ganges, Brah-

maputra, Indus and the Orinoco, only 20–25% of the total POC belong to the labile fraction. Remarkably, the rivers altered by human activities (constructions of dams and barrages) such as the Nile, Niger, Orange and the Indus have low labile organic carbon contents in their POC measured close to their mouths.

The distribution pattern of LPOC within the various groups given in Table 1 shows that, in general, for samples with TSS concentrations < 150 mg l<sup>-1</sup> the LPOC contributed to 22–46% (average of 35%) of the bulk POC. In those with TSS concentrations > 150 mg l<sup>-1</sup> its contribution was 11–18% (average 15%). Assuming equal contribution from other classes of labile organic matter (other than sugars and amino acids used here), the average values of LPOC in these two groups are 70 and 30%, respectively. The total POC fractions carried by these two classes of TSS concentrations are 29 and 202 × 10<sup>12</sup> g C yr<sup>-1</sup> (total POC 231 × 10<sup>12</sup> g C yr<sup>-1</sup>). The associated LPOC fractions are, respectively, 20.3 and 60.6 × 10<sup>12</sup> g C yr<sup>-1</sup> (total LPOC 80.9 × 10<sup>12</sup> g C yr<sup>-1</sup>). Thus, on average 35% of the organic carbon transported in river suspensions is labile and may become degraded in the aquatic sedimentary environment. Expressed differently, ~65% of the bulk POC in rivers (that is, 150 × 10<sup>12</sup> g C yr<sup>-1</sup>) is refractory, and has a chance to accumulate in marine sediments.

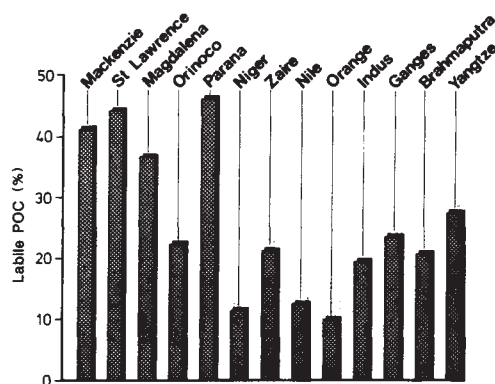
The assessed degradability from our data is lower than previous estimates on the loss or degradation of river-derived organic matter<sup>3,4</sup>. It may be argued that our estimate does not encompass all the organics that might eventually become degraded. However, we have given adequate consideration to contributions from other classes of labile organics in our calculations. Hence the higher degradability obtained in previous estimates is probably due to heavy reliance on the data obtained from rivers of temperate regions. The pattern of river sediment discharge to the modern ocean shows that most of the sediment input to sea takes place between 40° N and 12° S<sup>15,16</sup>. Data on lignin degradation products in fine suspensions from the Amazon River show them to be enriched in a fraction that is lignin-poor and suggest that the organic matter associated with fine suspensions there has undergone considerable decomposition in the terrestrial environment<sup>17</sup>. Thus the characteristics inferred from this study are valid for all the major rivers draining into tropical and subtropical seas: rivers that account for the bulk of POC transport. Biogeochemical processes occurring in soils, and in the flood plains and shallow lakes in the drainage area, lead to the transport of more biodegraded organic matter (that is, RPOC) by these rivers<sup>17,18</sup> (Fig. 2).

The low LPOC values found in the rivers altered by the construction of dams and barrages could result from retention



**Fig. 1** Relationship between total suspended solids (TSS) and particulate organic carbon (POC, as percentage of TSS) in world rivers. The bars indicate  $\pm 1$  s.d. (s) ranges about the respective mean POC contents ( $\bar{x}$ ). TSS concentration range (in mg l<sup>-1</sup>) for samples represented by each point is given on top of plot; n, number of samples





**Fig. 2** Distribution of labile particulate organic carbon (LPOC, expressed as the total percentage contribution of sugars and amino acids to total particulate organic carbon) in river suspensions from the rivers listed. For the Magdalena, Yangtze and Zaire rivers the LPOC values are averages (>2 samples) from one-time sampling. For the others, they are averages (>4 samples) representing at least one seasonal cycle.

of nutrients (lacustrine production and sedimentation) and/or biodegradation of organic matter within the man-made lakes. The retention of nutrients can further reduce the autochthonous (labile) input of organic matter in the lower reaches of these rivers<sup>14</sup>.

The extent of the influence of riverine RPOC in the marine environment will depend on physical (for instance mixing, sedimentation), and biogeochemical processes (for instance decomposition) in the oceanic water column. The available lignin and stable-carbon-isotope data from marine sediments<sup>19,20</sup> suggest that the influence of land-derived organic matter is restricted to nearshore environments. However, the largest sediment input to the modern ocean occurs from the main rivers of South-East Asia, which have depositional centres in well-developed deep-sea fans<sup>3,15</sup> extending several thousand kilometres offshore. Furthermore, indiscriminate use of stable carbon-isotope ratios for source identification is not an easy task, as wide variations in these ratios have been observed in materials derived from the same source (for instance marine plankton) depending on factors such as temperature<sup>22</sup> and their biochemical composition<sup>23</sup>.

Alteration of river-derived organics in the oceanic water column depends on their molecular nature, and the type of their association with mineral matter<sup>24</sup>. My data show that most of the organic matter is poor in labile constituents and, to judge from the nature of the mineral fraction<sup>25</sup>, associated with clay. Both these factors render the organics less susceptible to further degradation. Once in the oceanic water column they can be rapidly transferred to the sea floor packaged in large particle aggregates generated *in situ*<sup>26,27</sup>.

Today's riverine RPOC flux, though smaller than the other oceanic fluxes of particulate organic carbon<sup>28</sup>, is thus a significant factor in organic carbon removal in modern oceans. The available estimates of organic carbon burial in modern marine sediments (for instance ref. 4) suggest that its contribution can be significant. Assessing the land-derived component of modern marine sediments is of great relevance not only for understanding present-day marine organic carbon cycling, but also for palaeoproductivity studies based on organic carbon contents of ancient marine sediments.

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## Downward flux of particulate organic matter in the ocean: a particle decomposition paradox

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Oceanographers now recognize two distinct classes of particles in seawater, broadly categorized as suspended and sinking. The former class dominates the standing stock of particulate matter in the ocean and the latter class dominates the exchange between the surface waters and greater ocean depths<sup>1</sup>. The downward vertical flux of particulate organic matter (POM) in the open ocean exhibits a non-linear decrease with increasing water depth<sup>2-6</sup>, and greater than 75% of the net POM loss occurs in the upper 500 m of the water column<sup>6</sup>. Because sinking particles contain viable, metabolically active microorganisms<sup>7-12</sup>, the process of microbial decomposition is considered to be an important mechanism controlling POM flux. This model is consistent with the observed correspondence between POM flux and dissolved inorganic carbon concentrations<sup>13</sup>, and with the reported selective loss of biochemically labile compounds from sinking particles<sup>14-17</sup>. From our experiments, however, we conclude that the large sinking particles are, in general, poor habitats for bacterial growth and therefore unlikely sites for the active remineralization of organic matter. Our results require a shift in the emphasis of current ideas of particle decomposition from microbes attached to rapidly sinking particles to the microbial populations which are either free-living in the water column or attached to suspended (non-sinking) particulate matter.

**Table 1** Microbial ATP in association with sediment trap-collected particles at a variety of sites in the north Pacific Ocean

| Location (date;<br>deployment period)                        | Trap<br>depth<br>(m) | Shipboard/<br><i>in situ</i>                        |                                                |                                             |
|--------------------------------------------------------------|----------------------|-----------------------------------------------------|------------------------------------------------|---------------------------------------------|
|                                                              |                      | <i>In situ</i><br>ATP<br>( $\mu\text{g per trap}$ ) | Shipboard<br>ATP<br>( $\mu\text{g per trap}$ ) | <i>in situ</i><br>ATP<br>( $\times 100\%$ ) |
| Manzanillo, Mexico<br>18° N, 108° W<br>(10/81; 22 days)      | 30                   | 76.7                                                | 1.4                                            | 2                                           |
|                                                              | 120                  | 25.0                                                | 2.1                                            | 8                                           |
|                                                              | 200                  | 10.9                                                | 2.5                                            | 23                                          |
|                                                              | 400                  | 0.8                                                 | 1.0                                            | 125                                         |
| Pt Sur, California<br>36°16' N, 122°57' W<br>(6/82; 6 days)  | 100                  | 42.1                                                | 5.7                                            | 14                                          |
|                                                              | 300                  | 27.9                                                | 1.4                                            | 5                                           |
| Hawaii<br>28° N, 155° W<br>(8/83; 33 days)                   | 150                  | 34.0                                                | 2.5                                            | 7                                           |
|                                                              | 300                  | 16.2                                                | 1.8                                            | 11                                          |
|                                                              | 500                  | 15.8                                                | 1.7                                            | 11                                          |
| Pt Sur, California<br>35°40' N, 123°50' W<br>(6/84; 13 days) | 50                   | 30.8                                                | 2.7                                            | 9                                           |
|                                                              | 100                  | 9.1                                                 | 1.3                                            | 14                                          |
|                                                              | 225                  | 1.5                                                 | 0.5                                            | 33                                          |
|                                                              | 475                  | 0.7                                                 | 0.1                                            | 14                                          |
| North Pacific Ocean<br>35° N, 128° W<br>(6/84; 22 days)      | 130                  | 3.4                                                 | 0.7                                            | 21                                          |
|                                                              | 300                  | 1.0                                                 | 0.1                                            | 10                                          |
|                                                              | 600                  | 0.1                                                 | 0.1                                            | 100                                         |
| North Pacific Ocean<br>33° N, 139° W<br>(6/85; 22 days)      | 150                  | 3.7                                                 | 0.3                                            | 8                                           |
|                                                              | 275                  | 1.0                                                 | 0.1                                            | 10                                          |
|                                                              | 600                  | 0.2                                                 | 0.1                                            | 50                                          |

*In situ* ATP concentrations were determined in sediment traps pre-charged with a  $\text{H}_3\text{PO}_4$ -NaCl solution<sup>18</sup> and shipboard ATP was measured in unpreserved sediment trap collections<sup>11</sup> after deployment periods indicated above.

Sinking particles were collected during the VERTEX research programme at seven stations in the north Pacific Ocean using a free-floating MULTITRAP system<sup>2</sup>. Three different sediment trap solutions were used in this study: a NaCl-formalin solution for the collection of particulate organic carbon (POC) and nitrogen (PON)<sup>2</sup>, a  $\text{H}_3\text{PO}_4$ -NaCl solution for the *in situ* extraction of adenosine-5'-triphosphate (ATP)<sup>18</sup>, and a sterile seawater salt solution for the assessment of *in situ* microbial growth<sup>11</sup>. Nitex screens (335  $\mu\text{m}$  mesh) were placed at the base of each sediment trap baffle to minimize contamination by actively mobile zooplankton, which would have seriously compromised data interpretation.

Our experiments were designed to test the hypothesis that microbial (in particular, bacterial) decomposition of sinking organic matter is responsible for maintaining the observed profile of POM flux beneath the euphotic zone. Specifically, we endeavoured to evaluate two ecological predictions which would

be consistent with this hypothesis: (1) source term POM (the initial sinking particle pool) contains a low biomass of metabolically-active microorganisms which multiply as particles age (and sink); (2) microorganisms associated with particulate materials collected at the base of the euphotic zone and aged *in situ* consume POC and PON.

At every VERTEX station we observed a significant decrease in the flux of POM (both POC and PON) with increasing water depth (data summarized in refs 5 and 6), a result which is consistent with existing POM flux models<sup>3,4</sup>. The total ATP content of the sinking POM when the particles entered the sediment traps (that is, *in situ* extracted ATP) also decreased with increasing water depth (Table 1). This decrease in particle ATP content could represent either a decrease in total microbial biomass (assuming a constant microbial ATP:biomass-C relationship<sup>19</sup>) or a decrease in the microbial ATP:biomass-C ratio with increasing water depth. Direct microscopic measurements of bacterial and zooflagellate biomass made during the VERTEX 5 field experiment<sup>12</sup>, however, confirmed that the ATP:biomass-C relationship does not change substantially over the depth range of these *in situ* observations. Consequently, we conclude that there exists a decrease in the flux of living microorganisms with depth beneath the euphotic zone. This observed decrease in particle-associated microbial biomass does not support the original hypothesis regarding the role of microbial decomposition as a primary factor controlling downward POM flux in the ocean. Furthermore, the greatest loss of ATP per unit water depth was consistently observed for the depth stratum immediately beneath the euphotic zone. This is precisely the location where the microbial decomposition model would predict the most intense microbial metabolic activity and growth because the sinking particles would be closest to their source in the surface waters of the ocean.

Several *in situ* microbial growth experiments were performed during the VERTEX programme to complement the *in situ* extracted ATP data. Replicate sediment traps containing sterile seawater salt solutions were used to collect sinking particles which were then allowed to age *in situ* for the duration of the deployment period. The biomass of the particle-associated microbial assemblages which enter these 'live' traps could either increase, decrease or remain the same, depending upon the availability of reduced carbon and energy sources required for net microbial biosynthesis. Following the recovery of the sediment traps the ATP in these unpreserved traps was immediately extracted and these 'shipboard extracted' ATP concentrations were compared with the ATP values derived from the *in situ* extractions, discussed above. The results of this comparison indicate that for all stations investigated, there was net death (that is, net loss of ATP) during the respective incubation periods (Table 1). For the samples collected at the base of the euphotic

**Table 2** Effects of *in situ* incubation on the growth of bacteria and zooflagellates and on the decomposition of POC and PON associated with sinking particles

| Trap<br>depth<br>(m) | Trap<br>treatment | POC<br>(mg per trap) | PON<br>(mg per trap) | C:N<br>(w/w)       | Shipboard<br>ATP<br>( $\mu\text{g/trap}$ ) | Shb./ <i>In situ</i><br>ATP<br>( $\times 100\%$ ) | <sup>3</sup> H-adenine incorporation<br>( $\mu\text{Ci per trap}$ ) |     |         |
|----------------------|-------------------|----------------------|----------------------|--------------------|--------------------------------------------|---------------------------------------------------|---------------------------------------------------------------------|-----|---------|
|                      |                   |                      |                      |                    |                                            |                                                   | RNA                                                                 | DNA | protein |
| 80                   | live              | 2.72 ( $\pm 0.73$ )  | 0.46 ( $\pm 0.14$ )  | 6.0 ( $\pm 0.6$ )  | 1.2                                        | 27                                                | 47.3                                                                | 3.5 | 1.1     |
|                      | live + thiram     | 3.34 ( $\pm 0.48$ )  | 0.51 ( $\pm 0.11$ )  | 6.5 ( $\pm 0.5$ )  | 1.7                                        | 37                                                | 40.8                                                                | 2.4 | 0.7     |
|                      | formalin          | 3.19 ( $\pm 0.35$ )  | 0.48 ( $\pm 0.08$ )  | 6.7 ( $\pm 0.5$ )  | —                                          | —                                                 | —                                                                   | —   | —       |
| 175                  | live              | 1.30 ( $\pm 0.36$ )  | 0.14 ( $\pm 0.04$ )  | 9.2 ( $\pm 0.4$ )  | 0.4                                        | 17                                                | 1.9                                                                 | 0.2 | 0.1     |
|                      | live + thiram     | 1.17 ( $\pm 0.20$ )  | 0.14 ( $\pm 0.04$ )  | 10.3 ( $\pm 2.6$ ) | 0.4                                        | 17                                                | 3.9                                                                 | 0.8 | 0.1     |
|                      | formalin          | 1.67 ( $\pm 0.13$ )  | 0.21 ( $\pm 0.06$ )  | 10.6 ( $\pm 3.4$ ) | —                                          | —                                                 | —                                                                   | —   | —       |

Sediment traps were deployed at two depths for a period of 12 days off Pt. Sur, CA (35°40' N; 123°50' W) during April 1985. All traps were set with 335  $\mu\text{m}$  screens to eliminate contamination by large zooplankton. The trap solutions include:  $\text{H}_3\text{PO}_4$ , for the *in situ* extraction of ATP<sup>18</sup>; formalin, for the *in situ* preservation of POC and PON<sup>2</sup>; and two sterile seawater solution traps, without preservative, for the measurement of *in situ* microbial growth and organic matter decomposition<sup>11</sup>. The decomposition measurements were carried out both with and without thiram in order to evaluate the role of protozoans in particle decomposition<sup>12</sup>. The *in situ* extracted ATP concentrations were 4.62 and 2.47  $\mu\text{g ATP per trap}$  for sediment traps deployed at 80 and 175 m, respectively.



zone the extent of net community death ranged from 79.5% (North Pacific Ocean, 6/84) to 98.2% (Manzanillo, Mexico, 10/81). Again, these data are inconsistent with the hypothesis that microbial growth and metabolism are responsible for controlling the change in POM flux with depth in the upper water column. If the process of active microbial decomposition was important we would have expected to observe an increased ATP flux with depth and a net positive increase in ATP during the *in situ* sample incubations, both resulting from the hypothesized rapid growth of microorganisms on the sinking particles.

Additional experiments were designed to measure directly the decomposition of sediment trap-collected POC and PON during *in situ* incubation (Table 2). The results indicate that there was no difference between the amount of organic matter collected and preserved *in situ* and that which remained in the sediment traps after the particulate materials were allowed to decompose for 12 days in replicate unpreserved sediment traps either with or without the protozoan inhibitor thiram<sup>12</sup>. The vertical POM flux profile measured at this station would have required a net loss of 60–65% of the POC and PON per day, assuming a sinking rate of 100 m per day. In agreement with the data presented in Table 1, there was no evidence for an increase in microbial biomass with depth or for *in situ* microbial growth on the intercepted particulate materials. The *in situ* metabolic activity of the resident microbial assemblages, as determined by the incorporation of <sup>3</sup>H-adenine, indicated a substantial decrease in total metabolic activity with increasing water depth for particles collected in the upper 175 m of the water column, and an order of magnitude decrease in the ATP(biomass)-specific rate of <sup>3</sup>H-adenine assimilation.

During the VERTEX 5 field experiment (July–August 1985) we also measured POC and PON flux in 335 µm Nitex-screened sediment traps, in addition to ATP flux, to evaluate the relationship between living and non-living particulate carbon flux in the ocean (Table 3). The biomass (living) carbon flux extrapolated from the *in situ* extracted ATP measurements indicates that the majority of the sinking particulate carbon leaving the euphotic zone (93%, 54.1% and 91.6% for VERTEX-5 STA. 1, 2 and 3, respectively; Table 3) was composed of living microbial biomass. In addition, the ATP content of the collected particulate materials decreased with increasing depth to a greater extent than the content of POC (Table 3). These data imply that the ratio of biomass carbon to total particulate carbon decreases by a factor of 5–10 in the upper 2000 m of the water column. These observations are inconsistent with our initial microbial decomposition model. If rapid microbial growth was responsible for controlling the POC flux profile in the upper 500 m of the water column, we would have expected to observe an increasing trend in microbial biomass per unit POC as the particles were decomposed. Based upon these results we must conclude that sinking particles are, in general, poor habitats for bacterial growth and must be considered unlikely sites for the active remineralization of organic matter. This conclusion, though inconsistent with current hypotheses regarding the decomposition of large particles in the sea, is consistent with direct measurements of heterotrophic microbial activity on freshly collected crustacean faecal pellets<sup>20</sup> and macroscopic organic aggregates (marine snow)<sup>21,22</sup> and with the long time periods (1,500 days) which have been estimated for the microbially-mediated turnover of sediment-trap-collected POC<sup>8</sup>.

Our current models of organic matter diagenesis and nutrient regeneration require a balance between the downward flux of biogenic particles and the upward diffusion-advection of dissolved inorganic constituents. These large scale oceanic processes are ultimately dependent upon the production and remineralization of POM through the combined activities of diverse assemblages of marine microorganisms. If the assumption of steady-state is made, then a balance between the downward flux of POC and upward mixing can be used to predict the vertical distribution of dissolved inorganic carbon<sup>13</sup>. The collective data

**Table 3** Relationship between the flux of total particulate organic carbon (POC) and biomass-carbon in the north Pacific Ocean (VERTEX 5 experiment)

| Location and deployment period                          | Trap depth (m) | Biomass-C flux (mg C m <sup>-2</sup> per day) | POC flux (mg C m <sup>-2</sup> per day) | Per cent living |
|---------------------------------------------------------|----------------|-----------------------------------------------|-----------------------------------------|-----------------|
| Pt Sur California<br>35°40' N, 123°50' W<br>(22 days)   | 50             | 152                                           | 164                                     | 93              |
|                                                         | 100            | 45.0                                          | 49.2                                    | 92              |
|                                                         | 225            | 7.5                                           | 24.8                                    | 30              |
|                                                         | 475            | 3.4                                           | 9.0                                     | 38              |
|                                                         | 900            | 1.7                                           | 8.5                                     | 20              |
|                                                         | 1,500          | 1.6                                           | 8.7                                     | 18              |
|                                                         | 2,000          | 1.2                                           | 7.1                                     | 17              |
| North Pacific Ocean<br>35° N, 128° W<br>(22 days)       | 130            | 9.8                                           | 18.1                                    | 54              |
|                                                         | 300            | 2.9                                           | 6.1                                     | 48              |
|                                                         | 600            | 0.4                                           | 6.2                                     | 7               |
|                                                         | 750            | 0.5                                           | 5.9                                     | 9               |
|                                                         | 1,000          | 0.4                                           | 4.7                                     | 9               |
|                                                         | 1,500          | 0.2                                           | 5.0                                     | 4               |
|                                                         | 2,000          | 0.3                                           | 4.1                                     | 7               |
| North Pacific Ocean<br>33°06' N, 139°34' W<br>(13 days) | 150            | 10.9                                          | 11.9                                    | 92              |
|                                                         | 275            | 2.9                                           | 7.8                                     | 37              |
|                                                         | 600            | 0.6                                           | 4.9                                     | 12              |
|                                                         | 750            | 0.5                                           | 3.3                                     | 15              |
|                                                         | 1,000          | 0.5                                           | 3.3                                     | 15              |
|                                                         | 1,500          | 0.4                                           | 2.4                                     | 17              |
|                                                         | 2,000          | 0.1                                           | 2.0                                     | 5               |

Three separate sets of sediment traps were deployed during June–July 1984 in a transect from Pt Sur, California, to a station approximately 1,600 km offshore. All sediment traps were configured with screens and contained a sterile seawater solution without preservative. Following recovery, subsamples of each trap were taken for shipboard ATP and for POC (ATP data are given in Table 1). The shipboard ATP values were extrapolated to biomass-C assuming a C: ATP ratio of 250 (ref. 19).

presented here, however, indicate that the relationship between the downward flux of particulate matter and the regeneration of inorganic nutrients is most likely the result of an indirect coupling rather than being the direct result of microbial decomposition of sinking particles.

Two alternative processes can be invoked to explain the net loss of organic matter as particles sink. First, abiotic particle fragmentation (conversion of sinking particles to smaller non-sinking particles) and solubilization (conversion of sinking particles to dissolved constituents) may be important processes in controlling the net loss of particulate organic matter from the upper regions of the sea. Once the organic matter has been removed from the rapidly sinking particles, the microbial inhabitants of the upper mesopelagic zone (150–500 m) might be responsible for the oxidation of suspended particulate or dissolved organic matter resulting in the consumption of dissolved oxygen, the production of dissolved inorganic carbon and the regeneration of nitrate and phosphate. Although this model requires that heterotrophic microorganisms are ultimately responsible for the active remineralization processes, the site of active microbial growth and decomposition is shifted from microbes attached to rapidly sinking particles to the microbial populations which are either free-living in the water column or attached to suspended (non-sinking) particulate matter. An alternative model invokes particle ingestion by mid-water zooplankton and micronekton as the mechanism responsible for controlling the flux of POM. Decomposition of the particulate matter would then take place within the intestinal tracts of these animals, most likely as a result of the activities of psychrophilic and barophilic bacterial populations<sup>22, 24</sup>. These two decomposition models need not be mutually exclusive.

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## Major role of bacteria in biogeochemical fluxes in the ocean's interior

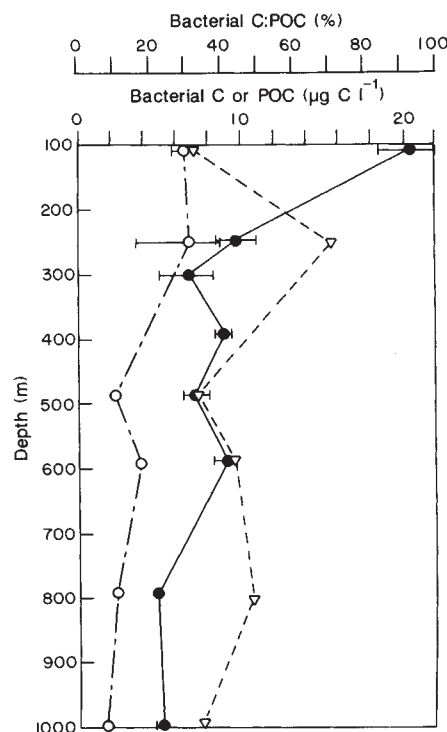
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**Spatial and temporal patterns in the flux of sinking organic matter are central to the understanding of elemental dynamics and food-web energetics in the global ocean<sup>1-3</sup>. Heterotrophic bacteria have been shown to play a part in the decomposition of large, rapidly sinking organic particles within and below the euphotic zone<sup>4-8</sup>. These previous studies suggest that decomposition by attached bacteria can explain only a trivial fraction of the observed decrease in the flux of organic matter with increasing depth. We report here that free-living bacteria, rather than the particle-feeding zooplankton, are the principal mediators of particle decomposition in the central north Pacific gyre and the eutrophic Santa Monica basin. We suggest that bacterial growth in the mesopelagial gives rise to the large-scale production of fine (0.3-0.6  $\mu\text{m}$ ), non-sinking particles at the expense of large, rapidly sinking particles. Our results have implications for models of biogeochemical dynamics of organic particles and surface-reactive materials such as radionuclides in the ocean's interior<sup>3,9</sup>.**

During the PRPOOS (Plankton Rate Processes in Oligotrophic Oceans) cruise in the north Pacific gyre (28°45.5' N, 118°47.6' W) in August-September 1985, we used water-bottle sampling to obtain depth profiles. Similar profiles were obtained from the eutrophic Santa Monica basin (33°45.5' N, 118°47.6' W) in May 1986. We took two 0.8-km-deep profiles in the basin and eight profiles in the gyre (of which four were 1-km deep). We used seawater from the same bottle for determining the bacterial abundance, bacterial organic carbon (BOC), bacterial secondary production (BSP; by the thymidine incorporation method<sup>10</sup>), particulate organic carbon (POC), and related parameters. We took measurements in both the euphotic and aphotic zones, but here we discuss only the layer from just below the euphotic zone to the bottom of the profiles (gyre, 110-1,000 m; basin, 39-800 m; water depth was >4,000 m in the gyre and 920 m in the basin).

BOC in the gyre formed a surprisingly large fraction of the POC; the mean value of the BOC/POC ratio was 0.43 (see Fig. 1). These estimates are in fact conservative as the POC values



**Fig. 1** Depth profiles of POC (closed circles), BOC (open circles), and BOC:POC in the gyre (open triangles). Bars represent the range for duplicate samples in the case of POC, and in BOC profiles they show 1 s.d. BOC was calculated from cell counts and cell volume measurements by epifluorescence microscopy<sup>25,26</sup> and by using 20 fg C cell<sup>-1</sup> for <0.07  $\mu\text{m}^3$  bacteria<sup>11</sup>. POC was measured by filtering seawater samples on GF/F filters (effective retention = 0.7  $\mu\text{m}$ ) followed by analysis by a CHN (carbon, hydrogen, nitrogen) analyser<sup>27</sup>. A correction was made for loss of bacteria through GF/F filters<sup>11</sup>.

were corrected for maximum loss of bacteria through the GF/F (glass-fibre) filters during sample filtration for POC measurements<sup>11</sup>. Our values of POC are close to the upper limit of an extensive data set from a station in the central Pacific<sup>12</sup>, whereas our values of the abundance of cells are lower than those measured earlier at the same station<sup>13</sup>, and are well within the range for other open-ocean environments<sup>6</sup>. POC and bacterial abundances in the gyre were previously measured at 500 and 900 m<sup>13</sup>, from which we calculate a BOC/POC ratio of ~0.7 (after correcting POC values for loss of bacteria through GF/F filters). High BOC/POC ratios are probably a common feature that has simply gone unnoticed before now. Even in the euphotic zone of the gyre the ratio was  $0.4 \pm 0.15$  ( $n = 15$ ). The corresponding value in the basin was  $0.43 \pm 0.19$  ( $n = 17$ ). Such high ratios have implications for the size spectrum of POC and biogeochemical cycling pathways in the oceans.

Epifluorescence microscopy showed that <5% of the bacteria were attached to particles, and 98% of the cells passed through 1  $\mu\text{m}$  Nuclepore filters<sup>14</sup>. Free-living bacteria were probably not released via disintegration of fragile particles during sampling because particle-attached bacteria are generally large (0.3-1.0  $\mu\text{m}^3$ )<sup>6,15</sup>, whereas in our samples cells as large as 0.1  $\mu\text{m}^3$  were rare. Size fractionation after incubation with [<sup>3</sup>H]thymidine generally showed >95% of the incorporation in bacteria passing through the 1  $\mu\text{m}$  filters. Recently it has been shown that marine aggregates harbour dense bacterial populations<sup>6</sup>, but these populations are few in number and contain a trivial fraction (<0.5%) of the bacterial assemblage. In view of these facts we believe that >95% of the mesopelagic bacteria in our profiles were free living, and were responsible for most of the bacterial production.

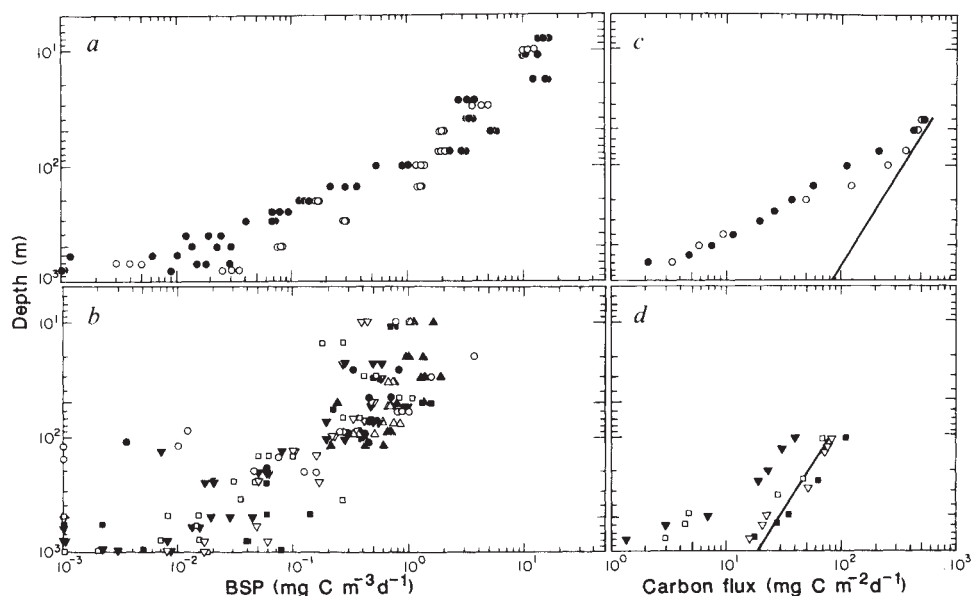
We measured bacterial production rates in the aphotic zones



**Fig. 2** *a, b*, Depth profiles of bacterial secondary production (BSP). *a*, Two profiles (open and filled circles) in the Santa Monica Basin (33°45.5' N, 118°47.6' W), taken 2 days apart in May 1986. *b*, Eight depth profiles (of which four are 1-km deep) in the central north Pacific gyre (28° N 155° W), represented by different symbols, taken during August–September 1985. All replicate sample measurements are shown. *c, d*, Depth versus  $F_B$  ( $=2 \int_{z_B}^z \text{BSP } dz$ , where  $z_B$  = depth at bottom of the profile and  $z$  = depth of integration); data points in *c* (gyre) and *d* (basin) are derived from BSP values in *a* & *b* respectively. Solid lines in *c* and *d*: depth versus sinking carbon flux calculated on the basis of local primary production and the sinking-flux model of Betzer *et al.*<sup>16</sup>. Symbols represent different profiles.

**Methods.** Seawater samples were collected with cleaned Go-Flo bottles

or Niskin bottles on CTD (conductivity-temperature-depth) rosette in depth-profiles to 1 km (gyre) and with Niskin bottles to 0.8 km (basin). BSP was measured by the thymidine (TdR) incorporation method<sup>10</sup> at *in situ* temperature and surface pressure<sup>28</sup>. BSP was calculated from TdR incorporation rates by using a conservative conversion factor of  $1.18 \times 10^{18}$  cells per mole TdR incorporated in DNA<sup>29</sup>. [<sup>3</sup>H] in DNA as a percentage of [<sup>3</sup>H] in cold trichloroacetic acid precipitate in euphotic zone was 65.7%, decreasing with depth to 29% at 450 m and below; the decrease from below the euphotic zone to 450 m was described by regression equation: (% [<sup>3</sup>H] in DNA =  $60.924 - 0.0709 \times \text{depth (m)}$ ). The precision of BSP measurements in the aphotic zone was on average  $22.7 \pm 14.2\%$  (mean  $\pm$  1 s.d.;  $n = 23$ ). Primary production was measured by the <sup>14</sup>C method in the gyre by Marra and Heineman<sup>30</sup> and in the basin by Eppley.



of the two study areas to address the following questions. (1) Are bacteria quantitatively important in the use of particulate organic matter (POM) flux? (2) Assuming steady-state conditions (growth = loss), how rapidly is the bacterial assemblage turned over due to various causes, including scavenging by rapidly sinking particles and by predation? (3) Does bacterial production reflect differences in the magnitude of surface primary production (PP) (456 and 1,254 mg C m<sup>-2</sup> day<sup>-1</sup> in the gyre and basin, respectively; Table 1)? Aphotic-zone integrated BSP was 39 mg C m<sup>-2</sup> d<sup>-1</sup> in the gyre and much higher (253 mg C m<sup>-2</sup> day<sup>-1</sup>) in the basin (Table 1). Clearly, the magnitude of BSP reflected the sinking flux out of the euphotic zone. Bacterial abundance in the aphotic zones of the two areas was comparable (data not shown); thus the 6-fold difference in BSP reflects higher specific growth rates (and assemblage turnover rates) in the basin. Although sinking flux (estimated from PP and Betzer's model<sup>16</sup>) at 800 m in the basin and 110 m in the gyre were comparable, BSP at 800 m in the basin was ~10 times lower than BSP at 110 m in the gyre (Fig. 2). Thus, BSP not only reflects the magnitude of C flux but also reflects properties that affect its bacterial use; factors such as packaging, size spectrum, and the chemical composition of sinking organic matter are probable modifiers of the depth variation of bacterial decomposition of sinking flux. For instance, high abundance of surface phytoplankton in the basin may support the production of large, compact, rapidly sinking fecal pellets<sup>17</sup> not significantly disintegrated and solubilized in the lower mesopelagic zone, although they may contain nutrients for bacteria.

We estimated C use by bacteria conservatively on the basis of BSP by assuming 50% carbon assimilation efficiency<sup>18</sup>. Integrated C flux into bacteria ( $F_B$ ) for the aphotic zone in the gyre and the basin was 78 and 507 mg C m<sup>-2</sup> day<sup>-1</sup>, respectively (Table 1;  $F_B$  may be somewhat overestimated if some BSP was released as dissolved organic matter (DOM) and re-used by bacteria<sup>19</sup>). As discussed,  $F_B$  mainly represents C flux into free-living bacteria. We now compare  $F_B$  with PP, new production (NP)<sup>20</sup>, and sinking flux ( $F$ )<sup>16</sup> to consider  $F_B$  in the context of sinking-flux biodynamics. Bacteria in the aphotic zone used sinking flux equivalent to 17% (gyre) and 40% (basin) of the

surface PP ( $F_B/PP = 0.17$  and  $0.40$ , respectively; Table 1). As  $F < PP$ , these values are also minimum estimates for  $F_B/F$ , that is the fraction of sinking flux used by bacteria. To estimate  $F_B/F$  more realistically we calculated  $F$  by Eppley and Peterson's new-production model<sup>20</sup> and by Betzer's carbon-flux model<sup>16</sup>.  $F_B/F$  calculated from the Eppley and Peterson model was 0.41 (gyre) and 0.81 (basin) whereas Betzer's model gave a value of 0.98 (gyre) and 0.80 (basin). Thus,  $F_B/F$  calculated from two conceptually and methodologically different models for determining  $F$  leads us to conclude that bacteria, rather than particle-consuming animals, were the main users of sinking C flux. Importantly, this result requires that a large part of the sinking organic matter must become DOM and thereby becomes accessible to the free-living bacteria. Consequently, this process represents a large-scale production of fine (0.3–0.6 µm diameter), non-sinking POC (that is, free-living bacterial cells) at the expense of large, rapidly sinking particles. As  $F_B$  explains a large fraction of  $F$ , it could be useful in making independent, minimum estimates of  $F$ .  $F_B$  determination is easy, rapid and

**Table 1** Carbon flux parameters for the gyre and the basin

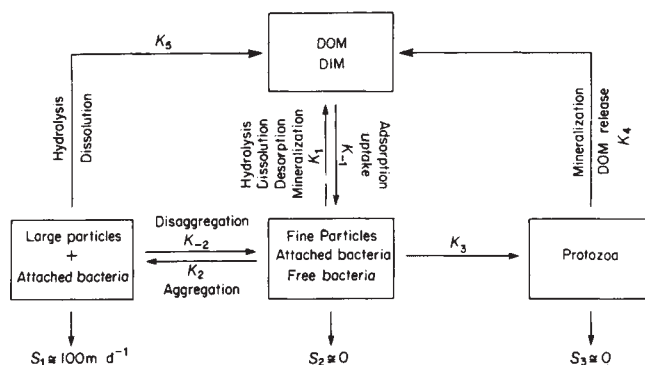
|                                               | Gyre                      | Basin                  |
|-----------------------------------------------|---------------------------|------------------------|
| PP (mg C m <sup>-2</sup> d <sup>-1</sup> )    | 456*                      | 1254†                  |
| Euphotic depth (m)                            | 110*                      | 39                     |
| $F$ (mg C m <sup>-2</sup> d <sup>-1</sup> )   | 79                        | 634                    |
| $F_B$ (mg C m <sup>-2</sup> d <sup>-1</sup> ) | 78 ± 34 (110–1,000 m)     | 507 ± 22 (39–800 m)    |
| $F_B/PP$                                      | 0.17 ± 0.07               | 0.40 ± 0.02            |
| $F_B/NP$                                      | 0.41 ± 0.18               | 0.81 ± 0.04            |
| $F_B/F$                                       | 0.98 ± 0.42 (110–1,000 m) | 0.80 ± 0.03 (39–800 m) |

PP, primary production;  $F$ , flux of sinking organic matter entering the aphotic zone, calculated as in legend to Fig. 2;  $F_B$ , Integrated  $2 \times$  BSP from the top of the aphotic zone to the bottom of the profile (depth-intervals in parentheses); NP, new production, calculated on the basis of PP, as in Eppley and Peterson<sup>20</sup>.

\* Ref. 30.

† Eppley, R. W. *et al.* (unpublished manuscript).

**Fig. 3** Schematic diagram for pathways of sinking particulate organic matter (POM) flux, modified from the model of Bacon *et al.*<sup>3</sup>. DOM and DIM represent dissolved organic matter and dissolved inorganic matter, respectively.  $K_1$ – $K_5$  denote the rate constants for various flux pathways.  $S_1$ – $S_3$  denote the sinking rates of various particle classes. See text for details.



inexpensive. It could supplement the sediment-trap measurements of the magnitude and spatial and temporal (including depth) variation of  $F$ , and could allow for extensive coverage.

What mechanisms could explain the large-scale POM solubilization as revealed by the high  $F_B/F$  values? Attached bacteria could compete with particle-consumers by rapidly solubilizing POM via hyper-production of exohydrolases<sup>8</sup> (this makes POM inaccessible to the organisms that eat particles and releasing into the microenvironment both the colonizer's progeny and most DOM produced by hydrolysis. Counter-intuitively, although particle-attached bacteria grow in size, they generally do not multiply faster than the free-living ones; hence, their *direct* role in particle mineralization remains small (that is they play the critical role of particle solubilization, yet the DOM thus produced is mainly used by the free-living bacteria). This is consistent with the observation that <5% of thymidine incorporation is due to particle-attached bacteria. Karl *et al.* (personal communication) found that microbes (including bacteria) attached to sinking particles collected in sediment traps play a minor role in the decomposition of sinking POM; other biological or physicochemical processes must be mainly responsible for particle mineralization. According to our hypothesis, the colonizing bacteria become enlarged to permit rapid synthesis of exohydrolases. Enzyme hydrolysis may also contribute to particle disaggregation and fragmentation. This would explain how the POM of large particles might be solubilized by the attached bacteria and be used mainly by free-living bacteria. Also, bacteria and metazoan digestive enzymes passed into the fecal pellets may continue the hydrolysis as the fecal pellets descend through the water column; the resulting DOM could be used by both attached and free-living bacteria<sup>8</sup>. Although the mechanistic details of DOM production are not yet understood, our results indicate that sinking organic matter mainly follows the route: POM → (fragmentation) → DOM → free-living bacteria (Fig. 3).

Based on depth-averaged BSP and BOC, the turnover times of BOC ( $K_2$  and  $K_3$ ; Fig. 3) below the euphotic zone were 16 days (basin) and 65 days (gyre). BOC could be scavenged by sinking particles ( $K_2$ ) or mineralized *in situ* ( $K_3$ ), for instance by protozoan predation. Protozoa (2–5  $\mu\text{m}$ ), morphologically similar to the bacterivorous microflagellates in epipelagic<sup>21</sup> and mesopelagic<sup>22–23</sup> zones, were present ( $10^6 \text{ l}^{-1}$  just below the euphotic zone, decreasing to  $10^4 \text{ l}^{-1}$  at 1 km). We estimate that ~40% of BSP could be consumed by microflagellates, assuming they cleared  $20 \mu\text{l}$  seawater  $\text{cell}^{-1} \text{ h}^{-1}$  (ref. 21). Although direct demonstration and quantification of bacterivory is awaited, our observations are consistent with the presence of a mesopelagic microbial loop potentially capable of mineralizing part of the BSP.

Our results provide a biological explanation for the observations and the model of Bacon *et al.*<sup>3</sup> regarding particle and radionuclide fluxes (Fig. 3, modified from ref. 3 to incorporate a role for bacteria). These authors postulate that there is an unchanging pool of fine, non-sinking particles responsible for most radionuclide adsorption. They further envisage a con-

tinuous exchange of material between large and fine particles via disaggregation-aggregation as the particle sinks. Bacteria, being a significant fraction of POC, could comprise the unchanging pool of fine, non-sinking particles responsible for radionuclide adsorption (we estimate that ~90% of all organic particulate surface in our samples was due to bacteria); they can stick to suspended and sinking particles, resulting in the observed slow, seasonally varying vertical flux of fine particles (that is, bacterial biomass) and associated materials<sup>24</sup>. Our model (Fig. 3) emphasizes particle solubilization and bacterial production and complements Bacon's model by providing a mechanism for sustaining the presence of fine, non-sinking particles. It also provides a framework for explaining the recently observed patterns of sinking flux of Chernobyl nuclides<sup>9</sup>; surface-reactive nuclides would largely adsorb onto free bacteria which would then be swept down by large particles such as marine snow and fecal pellets. Thus, free bacteria, by virtue of being the dominant biotic surface and metabolic component in the mesopelagic, act as principal mediators of exchange equilibrium between dissolved and particulate phases involving both the sinking organic matter and associated surface-reactive materials. We conclude that the distributional patterns and growth dynamics of free bacteria could strongly influence the spatial and temporal patterns of biogeochemical cycling of materials in the ocean's interior.

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## Receptive fields in the body-surface map in adult cortex defined by temporally correlated inputs

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Receptive fields (RFs) obtained at specific cortical sites can be used to define a topographic map of the body surface in adult mammalian somatosensory cortex. This map is not static, and RFs at particular cortical sites can change in size and location throughout adult life. Conversely, the cortical loci at which a given skin surface is represented can shift hundreds of micrometres across the cortex in the koniocortical field, area 3b (refs 1–12). This plasticity suggests that RFs derive not from rigid anatomical connections, but by the selection of a subset of a large number of inputs. We have proposed that inputs are selected on the basis of temporal correlation<sup>11–15</sup>. Here we test this idea by altering the correlation of inputs from two adjacent digits on the adult owl monkey hand by surgically connecting the skin surfaces of the two fingers (the formation of syndactyly). This manipulation increases the correlation of inputs from skin surfaces of adjacent fingers. The striking discontinuity between the zones of representation of adjacent digits on the somatosensory cortex disappeared. These results support the hypothesis that the topography of the body-surface map in the adult cortex is influenced by the temporal correlations of afferent inputs.

Normal adult owl monkeys have a highly ordered representation of the contralateral body surface in the area 3b somatosensory cortical field in the anterior parietal neocortex (Fig. 1, top centre)<sup>16–19</sup>. In the sector of area 3b that represents the hand, the individual digits are represented as discrete, topographic entities and, as a rule, RFs are restricted to single fingers<sup>17</sup>. To test the hypothesis that representational discontinuities between digits in somatosensory cortical maps arise from the lack of temporal correlation of afferent inputs across these boundaries, we connected the skin of the adjacent digits D3 and D4 in each of three adult owl monkey hands. With this fusion of digital skin, the probability of synchronous stimulation of the skin of the two digits is greatly increased.

When we reconstructed the representation of the hand in the somatosensory map in cortical area 3b several months after digital fusion, the normal representational discontinuity between the digits had completely disappeared. An example is shown in Fig. 1 (results from the other two experiments were very similar). As shown, the region mapped in detail was centred around the locations of representation of the fused digits. RFs defined at 41 cortical loci extended onto both fingers across the line of fusion. In contrast, RFs found along the highly resolved outer border of the representation of the fused digits were, with one exception, restricted to a single digit. The incidence of double-digit receptive fields in these cases was significantly less than that derived from five detailed maps of digital representations in normal adult owl monkeys (chi-squared test with continuity correction, 151.482, d.f., 1,  $P < 0.0001$ ).

The internal topography of the map of the fused digits resembled that of a normal single digit. In a sequence of measurements across the representation of fused digits 3 and 4 in the cortex, RFs moved in an overlapping progression from the radial aspect of digit 3 to the ulnar aspect of digit 4, across a zone of two-digit RFs (Fig. 1). This sequence of overlapping

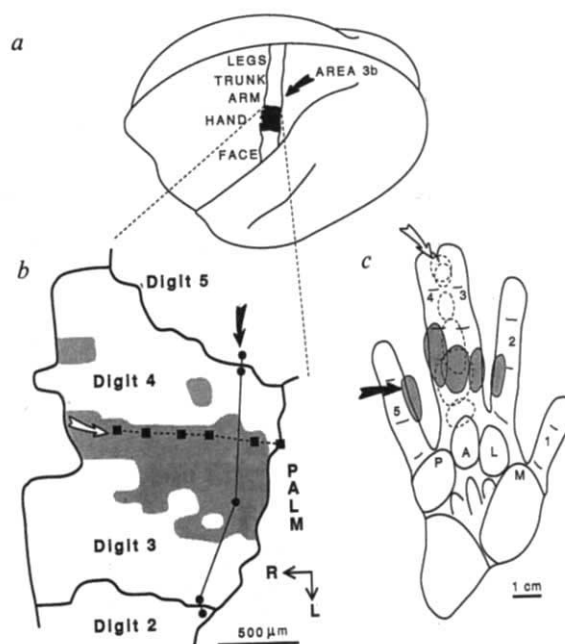
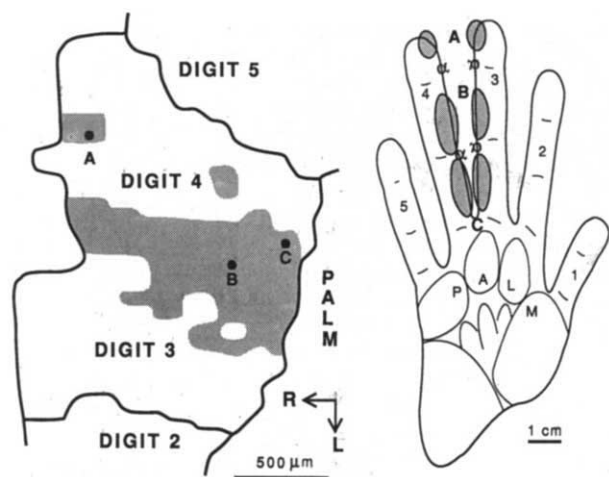


Fig. 1 Cortical representation after cutaneous fusion of digits 3 and 4. *a*, Dorsolateral view of adult owl monkey neocortex showing the location of the representation of the contralateral skin surface, including that of the hand (shaded), in cytoarchitectonic area 3b of the primary somatosensory cortex. *b*, Reconstruction of the cortical zones of representation of digits 3 and 4, and surrounding skin surfaces, five and a half months after surgical fusion of these digits. Area 3a borders this zone rostrally (to the far left). This detailed map was drawn from the identification of cutaneous receptive fields for cortical neurons defined at 209 microelectrode recording sites (mean interpenetration distance, 129  $\mu$ m). The shaded area marks the cortical region of double-digit representation following digit fusion. Nearly equal-threshold stimulation of surfaces of either one of the two fused digits evoked a cortical neuronal response at 41 sites within this zone. This two-digit representational zone ranged from 340  $\mu$ m to 1,000  $\mu$ m in width. In contrast, the borders of the representation of the fused digits with the adjacent digits 2 and 5 were abrupt, as is the case in normal hand representations in adult owl monkeys. Filled dots and squares mark recording sites in rostral-to-caudal (white arrow) and medial-to-lateral (black arrow) penetration sequences. RFs for neurons recorded at these marked locations are shown on the hand drawing, (*c*) (open and shaded regions respectively). Note the orderly progression of RFs across the zone of representation of the fused digits. R = rostral, L = lateral.

**Methods.** All hand surgical procedures were conducted using halothane/nitrous oxide general anaesthesia. The skin of ulnar digit 3 and radial digit 4 was incised along the midlateral line. Microscopic dissection and visualization of digital nerves ensured preservation of their integrity, and of the innervation of created dorsal and volar skin flaps. Both the dorsal and the volar skin margins of digits 3 and 4 were then sewn together with an 8-0 chromic suture on a microsurgical needle to minimize tissue trauma. A full arm cast in the position of function (elbow 90° flexion, wrist 30° dorsiflexion, metacarpophalangeal joints 90° flexion) was applied to the upper extremity for three to four weeks to protect the wound during healing. After removal of the cast, the fused digit pair had full range of motion. The fused digits were used by the monkey as a single digit; there was no indication of hand or digital neglect and no infections or post-operative complications. RFs were defined for neurons recorded in 209 parallel microelectrode penetrations introduced into the middle cortical layers in the relevant sector of the hand representational zone of area 3b (*b*). Cutaneous RFs were defined as those skin surfaces evoking a suprathreshold neural response with application of just-visible tactile stimulation with fine opaque glass probes. Many RFs were also defined with the use of von Frey hairs, which apply stimuli of constant force with each application. Under these conditions, the locations and sizes of RFs of area 3b neurons are very similar to those recorded in unanaesthetized monkeys<sup>20</sup>.



**Fig. 2** Double-digit receptive fields recorded after separation of cutaneously fused digits. *a*, Filled circles mark typical penetration sites within the cortical zone (shaded) at which equal-threshold or nearly equal-threshold responses were evoked by stimulation of the surfaces of both of the fused digits—and at which RFs were again defined immediately after the surgical separation of the fused digits. As in these three representative examples (at sites A, B, C), low-threshold cutaneous RFs were recorded on both digits (*b*) following separation at nearly all map locations sampled within this double-digit representational zone.

**Methods.** Under continuous barbiturate anaesthesia, maintained throughout and following the derivation of the initial map, an incision was made along the scar on the dorsal and volar surfaces of the fused digits and the digital nerves were clearly visualized. With microsurgical technique, the skin flaps were sewn along the midlateral line of each digit using interrupted 8-0 chromic suture on a microsurgical needle. In this way, the normal separation of digits was re-created. At this time, RFs were redefined at or near cortical sites where RFs previously had crossed the line of fusion.

RFs resembled that commonly seen in the detailed representation of single normal digits. Similarly, in sequential rostral-to-caudal microelectrode penetration sequences across the zone of two-digit representation, RFs progressed from the distal tip of the fused digits to its proximal base (Fig. 1). Results were consistent in all three experiments (Table 1).

One interpretation of these data is that a change in the peripheral, rather than central, nervous system is responsible for the disappearance of the representational discontinuity between fused digits. It is possible that, even though the skin was cut along the clearly demarcated lines of innervation of dorsal and volar digital nerves, significant peripheral nerve regeneration might occur spanning the scar line. Such nerve regeneration could result in RFs that extended onto the digital skin of adjacent fused digits. To test this possibility, the digits were separated microsurgically after completion of the cortical mapping procedure.

Despite the trauma of surgery and some unavoidable post-operative peripheral oedema, 20 of 21 cortical sites were still found to respond to stimulation of both of the formerly-fused digits following the separation of fingers in the case illustrated in Fig. 1 (see Fig. 2 for typical examples). In a second case, cutaneous RFs were defined on both formerly-fused digits for neurons at 14 of 15 sampled sites.

These controls provide a further proof that the RFs extending onto both of the two fused digits are not attributable to a mechanical artifact during receptive field mapping, and demonstrate that the breakdown of the representational discontinuity following digit fusion is due to a change in the central, rather than the peripheral, nervous system. Representational plasticity recorded in area 3b necessarily reflects any changes that occur

**Table 1** The location of receptive fields that map in the somatosensory cortex after cutaneous fusion of digits D3 and D4

| Case                               | Receptive field location |         |         | Total |
|------------------------------------|--------------------------|---------|---------|-------|
|                                    | D3 only                  | D3 + D4 | D4 only |       |
| 1 Cortical area (mm <sup>2</sup> ) | 0.91                     | 0.72    | 0.87    | 2.50  |
| No. of recording sites             | 45                       | 41      | 41      | 127   |
| 2 Cortical area (mm <sup>2</sup> ) | 0.94                     | 0.74    | 0.99    | 2.67  |
| No. of recording sites             | 46                       | 45      | 57      | 148   |
| 3 Cortical area (mm <sup>2</sup> ) | 0.94                     | 0.67    | 0.83    | 2.44  |
| No. of recording sites             | 34                       | 30      | 29      | 93    |

Data from three owl monkeys with fused digits showing cortical representational areas and the numbers of microelectrode penetration sites at which defined RFs were restricted to either digit 3 or digit 4 only, or extended across the line of fusion onto both digits (D3 + D4). Data were obtained 24, 34, and 13 weeks after surgical fusion of the digits in cases 1, 2 and 3, respectively. Measurements of area were made with a computer-controlled planimeter from cortical maps reconstructed from recordings from many, very closely spaced, microelectrode penetrations. RFs that extended onto both digits were identified over a large part (~28%) of the total cortical area of representation of digits 3 and 4 in all three cases.

at all levels in the lemniscal pathway from the dorsal column nuclei to the cortex, but we have argued previously that it must principally be accounted for by changes that occur at the cortical level itself<sup>11-15</sup>.

These experiments show that temporal correlations of inputs are important in the creation of neuronal RFs in the somatosensory cortex. When two digits were fused to produce a continuous body surface from a formerly discontinuous one, RFs were altered to create a progressively overlapping series of RFs like those recorded over the surfaces of any single digit. Thus the normal discontinuities in the cortical map between adjacent digits in area 3b of adult owl monkeys appear to be established by patterns of input activity, rather than by any sharply-demarcated limitation of their anatomical connections. We suggest that these correlation-based mechanisms underlie the representation of topographic detail in the somatosensory cortex. Their definition may provide a general model for the representation of 'selected' inputs within neocortical fields in the adult mammalian brain. It is of great interest to determine the specific mechanisms underlying correlation-based input selection, as these processes are undoubtedly central to understanding how the details of cortical representations become modified by use.

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# Immediate and chronic changes in responses of somatosensory cortex in adult flying-fox after digit amputation

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The somatosensory cortex of adult mammals has been shown to have a capacity to reorganize when inputs are removed by cutting afferent nerves or amputating a part of the body<sup>1-7</sup>. The area of cortex that would normally respond to stimulation of the missing input can become responsive to inputs from other parts of the body surface. Although a few animals have been studied with repeat recording<sup>1,2</sup>, no attempt has been made to follow the time-course of changes at cortical loci and the immediate effects of a small amputation have not been reported. We have followed the changes in response in the primary somatosensory cortex in the flying-fox following amputation of the single exposed digit on the forelimb. Immediately after amputation, neurons in the area of cortex receiving inputs from the missing digit were not silent but responded to stimulation of adjoining regions of the digit, hand, arm and wing. In the week following amputation, the enlarged receptive fields shrank until they covered only the skin around the amputation wound. The immediate response is interpreted as a removal of inhibition and the subsequent shrinking of the field may be due to re-establishment of the inhibitory balance in the affected cortex and its inputs.

Experiments were performed on adult little red flying-foxes (*Pteropus scapulatus*). The primary somatosensory cortex in this species has a similar organization to that reported previously for *P. poliocephalus*<sup>8</sup>. The forelimb digit representation in the primary somatosensory cortex was mapped initially using tungsten-in-glass electrodes and multi-unit recording techniques, and a small brush was used to stimulate the skin. The cortical representation was determined for the maximal area of skin to which neurons at a given recording site would respond; that is, the maximal receptive field, not just the area giving peak responses or the response area at threshold.

Four animals were studied using implanted electrodes (27 sites) allowing repeated determination of receptive fields in the period before amputation, immediately after amputation and at several times over the following 1-4 weeks. The immediate effects of amputation were also examined in a further three animals using tungsten-in-glass electrodes, which greatly increased the number of cortical sites that could be studied immediately after the operation. The results from the two groups of animals were consistent.

At 36 recording sites where the receptive fields lay far away from the digit, there was no significant change in field size or position after amputation. In contrast, most receptive fields that were originally restricted to the amputated digit had immediately enlarged to include parts of the remaining metacarpal and had extended on to the wing, arm or wrist (Fig. 1). Similar enlarged receptive fields were found at sites where the fields before amputation had spanned the amputation boundary (Fig. 2b, c). The receptive fields, apart from being larger than usual for this area, had apparently normal physiological properties and in no case did we find more than one field for a single recording site or fields that did not extend up to the amputation wound. At five recording sites (four from a single animal with implanted electrodes), however, we found no response at all to stimulation of the skin within a few hours of amputation. By the next recording session, 20 hr later, each of these sites had a large receptive field (Fig. 2a).

By the third day after amputation the area of all receptive fields that had been studied repeatedly was noticeably reduced (Fig. 2). This reduction in area continued for 7-8 days after amputation by which time a small receptive field, always extending to some part of the wound, remained. Seven receptive fields studied for more than 8 days showed little further change (Fig. 2c).

We have confidence that the implanted electrodes recorded stably from the same sites throughout the experiment from the results of a preliminary study in four normal animals, and from the pre-amputation recordings in this study (for example, Fig. 2c), in which the receptive field recorded by each electrode showed little change over a recording period of 3-9 weeks. This indicates both that the representation of the body surface is stable in the primary somatosensory cortex of normal animals and that the position of the electrodes did not change. The validity of the two major findings of this study (initial large post amputation receptive fields followed by gradual shrinking) does not, however, depend upon electrode stability, as all electrodes recorded similar effects.

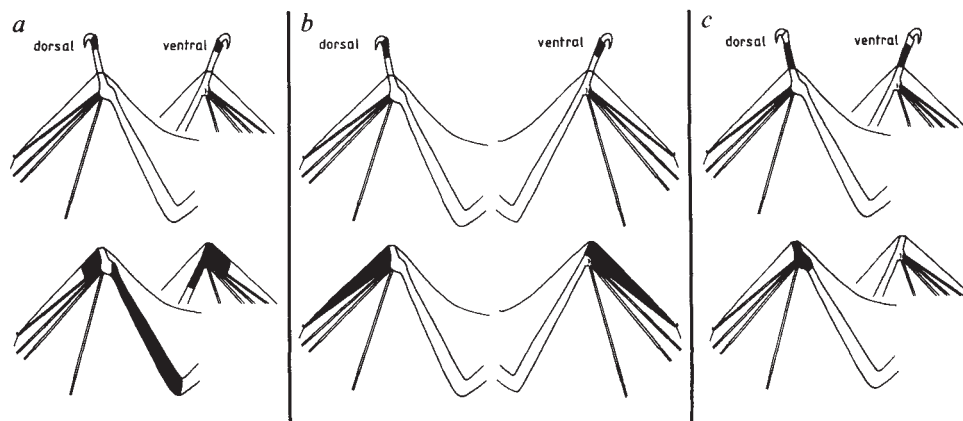
The expression of new cutaneous receptive fields after digit amputation is too immediate to be the result of new neural connections or other morphological changes. Instead it is likely that inhibitory effects which previously prevented the expression of existing inputs from areas around the initial field were reduced by the amputation. Throughout the somatosensory pathway lateral inhibition is thought to play a role in shaping receptive fields and in improving physiological resolution above that to be expected from the diffuse pattern of anatomical connections<sup>9</sup>. Most of the neurons that project from the cuneate nucleus to the thalamus<sup>10</sup> and from the thalamus to the cortex<sup>11</sup>, as well as about half the neurons in the primary somatosensory cortex, have fields with inhibitory surrounds. As in other centre-surround receptive fields, the inhibition extends throughout the excitatory fields and peaks at the same position. Hence removal of the digit containing all, or most, of the receptive field of a cortical locus also removes the dominant inhibitory input which may explain the immediate expression of the new, enlarged receptive fields that we observed. Similarly, the alteration of the excitatory-inhibitory balance by local iontophoresis of the GABA ( $\gamma$ -aminobutyric acid) antagonist bicuculline had been shown to increase dramatically the size of rapidly-adapting receptive fields in the primary somatosensory cortex.<sup>13</sup>

The other significant findings of these experiments are that the enlarged receptive fields seen after amputation progressively shrink and that the final receptive field is always adjacent to the wound. If expansion is caused by a removal of inhibition, shrinking implies gradual change in the excitatory/inhibitory balance. That the final receptive field is adjacent to the wound suggests that the final area gaining a net excitatory balance is the one that provides the greatest input (that is, closest to the original field). Furthermore, the shrinkage of the receptive fields as recorded in the cortex probably indicates that a change in balance is occurring at all lower stations of the pathway because, in normal animals, lateral inhibition appears to maintain but not to sharpen the resolution of incoming afferents.

Studies of the effects of partial denervation have also implied the existence of normally unexpressed inputs<sup>1-7,14</sup>. Some authors have suggested that new receptive fields are formed by minor inputs to a locus gaining increased efficacy<sup>1-3,5,6,15</sup> as a result of some 'driving force' in the new pattern of peripheral activities<sup>1,2,15</sup>. The advantage of using implanted electrodes to follow changes in receptive fields is the clear demonstration that final receptive fields are shaped from an immediately expressed, enlarged receptive field. This indicates an inherent viability in the normally unexpressed inputs and appears consistent with the hypothesis that their expression is dependent on the removal of inhibitory inputs. There is thus no need to invoke a change in excitatory synaptic efficacy to explain the effects of a small

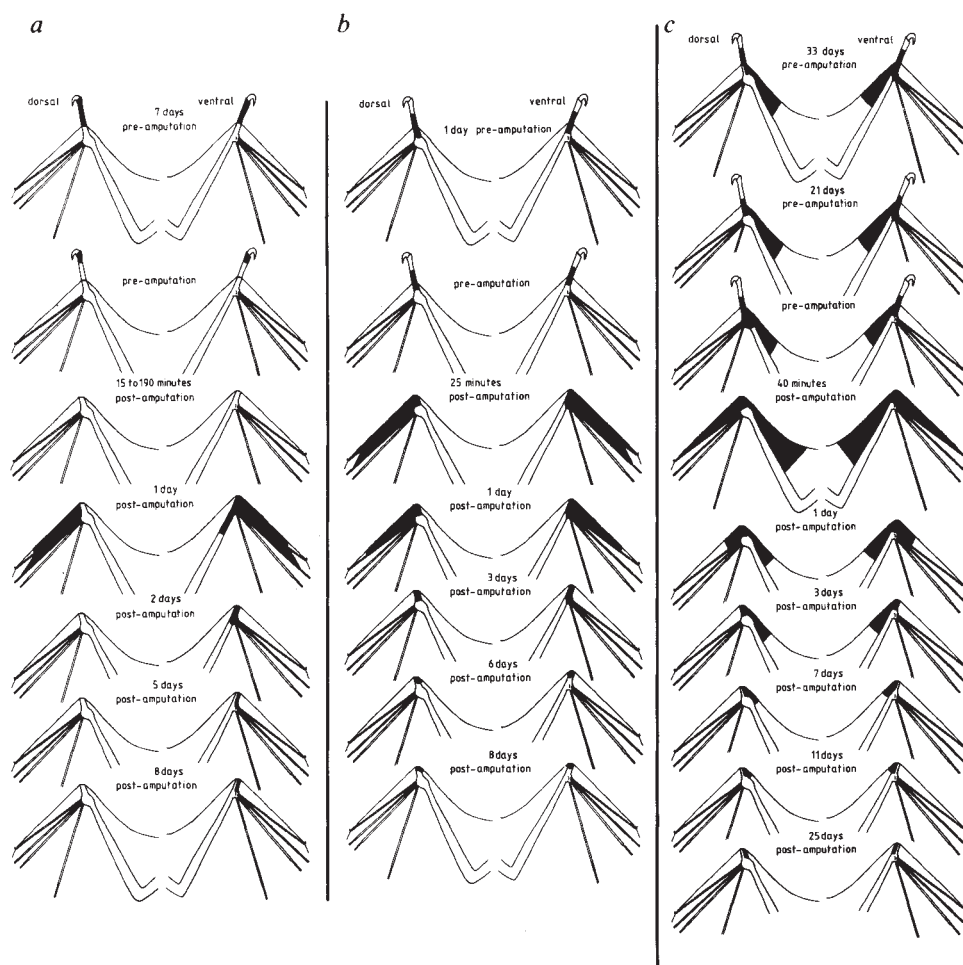
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**Fig. 1** Outline drawings of the dorsal and ventral surfaces of the forearm, wing, and digit 1 (D1), showing the multiunit, cutaneous receptive fields (black) recorded in primary somatosensory cortex at three loci (*a*, *b*, *c*; dorsal and ventral distinctions could not be made for receptive fields on the membranes of the wing). The digit was amputated and the receptive fields at each locus examined immediately post-amputation. Each locus was now responsive to cutaneous stimulation of a large area adjoining the wound to which there was originally no response. Amputation was carried out at the metacarpophalangeal joint where the prying and D1-D2 finger membrane insert on to D1. Medial and lateral blood vessels were tied and the wound was sutured with a single line of stitches. Surgery and initial recording were carried out under semi-sterile conditions. Animals were anaesthetized with ketamine (40 mg/kg) and xylazine (4 mg/kg) and a 5 mm by 5 mm area of cortex was exposed. In both groups of animals the forelimb D1 representation in SI was initially mapped using tungsten-in-glass electrodes (20  $\mu$ m exposed tip), oriented perpendicular to the surface, and multiunit recording techniques<sup>8</sup>.



**Fig. 2** Receptive fields in primary somatosensory cortex recorded through implanted electrodes before and at several times after digit amputation. *a*: Locus with original receptive field confined to the amputated region. The immediate effect of amputation was unlike that of the majority of such fields (Fig. 1), in that the locus was unresponsive, but a large field was found one day later. Over the following week the field shrank and finally the responsive area was restricted to the ventral surface of the forelimb digit D1 of the metacarpal joint adjacent to the amputation wound. *b* and *c*: Examples of loci with receptive fields which initially crossed the line of amputation. After amputation both show an immediate expansion of the field into originally unresponsive regions with subsequent shrinking toward the wound.

**Methods.** Implanted electrodes consisted of single strands of Teflon coated stainless steel (24  $\mu$ m diameter, Cooner Wire Co.) soldered to stainless steel pins, affixed to the back of the skull with dental acrylic which was also used to anchor the wire at the edge of the exposure. The wire was made into a loop, cut to length and the free end pushed perpendicular to the surface about 1 mm into the region of cortex, responding to stimulation of D1. The viability of, and receptive field of, each electrode was checked and, when 12-14 electrodes had been implanted, they were embedded in a layer of surgical-grade elastomer (Dow Corning), strengthened by a layer of acrylic. The electrodes gave good multi-unit recording and sometimes isolated single units, but the time over which they could be used varied. Some were apparently displaced by the setting elastomer whereas others remained effective for many weeks. During recording sessions the animals were again anaesthetized with ketamine and xylazine.



amputation on the receptive field organization in the primary somatosensory cortex.

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## Cholinergic-rich brain transplants reverse alcohol-induced memory deficits

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Alcohol-induced memory impairment in man has been attributed to deficiencies in subcortical noradrenergic<sup>1</sup> and cholinergic<sup>2,3</sup> systems, as well as to damage in midbrain structures<sup>4</sup>. Korsakoff's psychosis, a disease in which alcohol poisoning causes apparently irreversible memory defects, is characterized by lesions in cholinergic<sup>5</sup> and noradrenergic<sup>6</sup> nuclei and by a decrease in the activity of choline acetyltransferase<sup>7–10</sup> (ChAT) and the content of noradrenaline<sup>7</sup> (NA) in forebrain areas such as cerebral cortex and hippocampus, innervated by these nuclei<sup>11,12</sup>. Prolonged intake of ethanol in rodents similarly produces signs of noradrenergic<sup>1,13–15</sup> and cholinergic<sup>16,17</sup> deafferentation in the cortex and hippocampus, as well as persistent memory deficits<sup>17–19</sup>. To test whether alcohol-induced memory impairments depend on cholinergic deafferentation, we transplanted cholinergic-rich fetal basal forebrain cell suspensions<sup>20</sup> into the cortex and hippocampus of alcohol-treated rats. The substantial and persistent memory losses produced in our rats by ethanol intake were associated with an impairment of cholinergic function, and were reversed by cholinergic-rich transplants into cortex and hippocampus.

Male Sprague-Dawley rats were subjected to a high level of alcohol intake for 28 weeks and, after a further four weeks free of alcohol, were tested in an eight-arm radial maze<sup>21</sup> for memory function. Four of the eight arms were baited with reward and correct choices were defined either spatially (by extra-maze room cues, the 'place task') or non-spatially (by tactile/visual cues inserted into the arms of the maze, the 'cue task'). After two daily trials on each version, place and cue, of the maze all control rats over a period of 26 days reached a level of nearly error-free performance. Entries into any of the four arms which were never baited constituted reference memory (RM) errors; repeated entries into an arm that had initially been baited constituted working memory (WM) errors. Thus both reference memory<sup>22</sup>, involved in learning from the factors that remained constant in each task, and working memory<sup>22</sup>, involved in learning from the particular history of each trial, could be assessed for each task in a single block of trials. The ethanol-treated animals, however, after an initial period of improvement, stabilized at a much higher error rate (Fig. 1, left-hand panel). This impairment affected both working and reference memory and was more pronounced in the spatial task, a relative selectivity that is even more evident after only 18 weeks alcohol treatment<sup>16</sup>.

The alcohol-treated animals were randomly assigned to groups ( $n=10-12$ ) that received sham operations or bilateral grafts of fetal cell suspensions<sup>20</sup> prepared either from the cholinergic-rich septal/diagonal band area or from the cholinergic-poor hippocampus. Two implants were made bilaterally either into neocortex or into hippocampus or into both (Fig. 2). Testing of the animals with transplants, and of 16 non-alcohol-treated controls, in the memory task resumed 14 days after surgery with the same procedures as before, except that each animal received only 2 days' testing per week for 13 weeks. The non-alcohol treated controls continued to maintain an excellent performance, whereas the sham-operated alcohol-treated animals remained as impaired as before. Groups that received non-cholinergic implants did not differ from the rats that received sham operations. The rats with cholinergic-rich transplants, however, improved their performance over blocks of trials in a manner that depended on both the site of implant and the nature of the task (Fig. 1). After about the seventh week of testing the groups that received cholinergic implants in either cortex or hippocampus improved significantly in the place task in both reference and working memory relative to their corresponding non-cholinergic implanted control group. Moreover, improvement in the group that received the combined cholinergic implants in both cortex and hippocampus was significantly superior to the groups which received cholinergic implants in only one site. In the cue task neither single-site cholinergic implant led to a significant improvement over time relative to the corresponding non-cholinergic implanted controls, but the combined cholinergic implant group showed a significant improvement in both reference and working memory which emerged by the seventh week of testing. The group receiving implants in both cortex and hippocampus recovered on all four measures of memory almost to the level of the non-alcohol treated animals. It is unclear whether the apparently greater effect of the transplants on spatial than non-spatial memory merely reflects the greater deterioration of spatial memory caused by alcohol, or indicates a greater involvement of cholinergic systems in this type of memory.

At the end of behavioural testing one hemisphere was processed for histology, and the other for biochemistry. Routine histological examination did not reveal any of the diencephalic (midbrain) haemorrhagic pathology seen in the Korsakoff brain, though there was significant cell loss in the cholinergic nucleus basalis and septal/diagonal band complex (T. Arendt *et al.* in preparation). Histochemical staining for acetylcholinesterase (AChE) (Fig. 2) showed intense positive patches in the cholinergic-rich implants derived from ventral forebrain, with fibres sometimes extending into the host brain; in contrast, the implants derived from the cholinergic-poor hippocampus were almost AChE-negative. Biochemical analyses showed that in the non-implanted animals the alcohol regime caused significant reductions in regional brain concentrations (Table 1) of acetylcholine (ACh) and NA, ChAT activity, and capacity for ACh release and choline uptake. Brains with cholinergic implants revealed a 7–36% increase in the values of cholinergic parameters at the implant site, reaching 75–90% of the values in non-alcohol-treated controls. The highest recovery was found in ACh content and capacity for evoked ACh release, thus demonstrating the functional activity of the cholinergic transplants. The alcohol-induced changes in biochemical parameters persisted in the animals with cholinergic-poor transplants, not differing significantly from values in the sham operated rats or animals killed at the end of pre-operative testing. Noradrenaline content was unaffected by any of the transplants. Within the groups of animals that received cholinergic transplants, scores of pre-to post-operative improvement in memory correlated with ACh content at the site of the implant ( $r=0.99$  and  $0.89$ ,  $P<0.001$ , for the place and cue task, respectively), but not with NA content ( $r=0.08$  and  $0.11$ ).

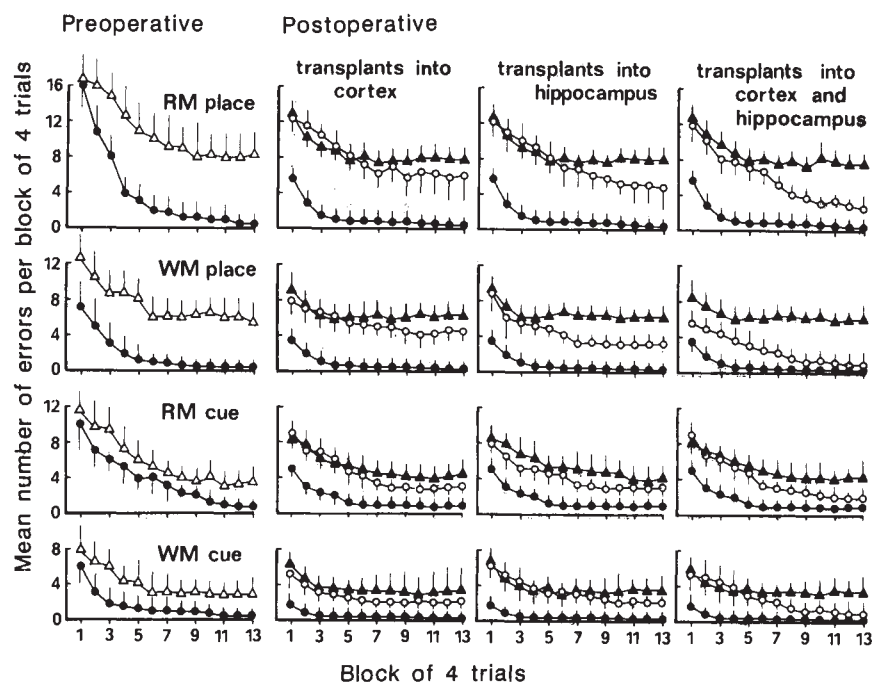
The memory deficit in the alcohol-treated animals showed no

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**Fig. 1** Mean numbers of errors cumulated over blocks of four trials in the eight-arm radial maze. In the spatial (place) version of the task, all arms were the same, correct arms being identified by location in the experimental room (2.4 × 2.7 m, lighted by an overhead fluorescent strip, with laboratory furniture and posters on the wall). In the non-spatial (cue) version, correct arms were identified by removable tactile/visual cues, such as perspex and sandpaper, laid on the floor and switched between spatial locations from trial to trial; the room furniture and lighting were as for the place version. In both versions, four arms were always baited with the reward (three drops 30% sucrose solution) and four never baited, entries into the latter constituting reference memory (RM) errors, repeated entries after the first into a baited arm constituting working memory (WM) errors. Each rat was given two trials on each version (place and cue) of the task in a single day, one version in the morning and the other in the afternoon. Left, pre-operative, daily testing (four trials over two days on each version).  $\Delta$ , alcohol-treated animals ( $N = 78$ ).  $\bullet$ , tap-water controls ( $N = 16$ ). Right, post-operative testing (four trials per week on each version).  $\circ$ , cholinergic transplants ( $n = 12$ ); see Fig. 2.  $\blacktriangle$ , non-cholinergic transplants ( $n = 10$ ).  $\bullet$ , tap-water controls ( $n = 16$ ). Pre-operatively, alcohol impaired performance on all four measures of memory, with values (degrees of freedom 1 and 92) equal to 790.5, 913.8, 159.1 and 460.9 for the main effects of the drug on place reference and working memory, cue reference and working memory respectively. Post-operatively, non-cholinergic implanted controls never differed from sham operated animals (data not shown). On the place task all groups with cholinergic implants improved significantly over time, relative to the corresponding non-cholinergic implanted controls: interaction with linear component of trial blocks for reference memory,  $F(1, 20) = 7.97$ , 10.84 and 44.68, for cortical, hippocampal and combined implants respectively; for working memory,  $F = 8.73$ , 17.71 and 27.31, respectively; the combined transplant group was significantly better (main effect of transplant site) than either the cortical-only group,  $F(1, 22) = 15.27$  and 184.48 for reference and working memory respectively, or the hippocampal-only group,  $F = 31.44$  and 64.27 respectively. On the cue task only the combined cholinergic transplant group improved more than the non-cholinergic transplant controls,  $F(1, 20) = 15.22$  and 16.57 for reference and working memory respectively. In all cases for cited  $F$ ,  $P < 0.01$ . Bars indicate standard deviations.

**Methods.** Male Sprague-Dawley rats (150–180 g) were given 20% (v/v) ethanol ( $n = 88$ ) or tap-water ( $n = 26$ ) for 28 weeks as the sole drinking fluid. Mean daily ethanol intake was 9.7 g per kg, and blood alcohol levels ranged from 40 (day) to 120 (night) mg per 100 ml. At the end of the 28 weeks the ethanol-treated animals were lighter in weight, though not significantly:  $533 \pm (\text{s.e.m.}) 14$  g versus  $551 \pm 15$ . The rats were fed for 1 h daily after behavioural testing.



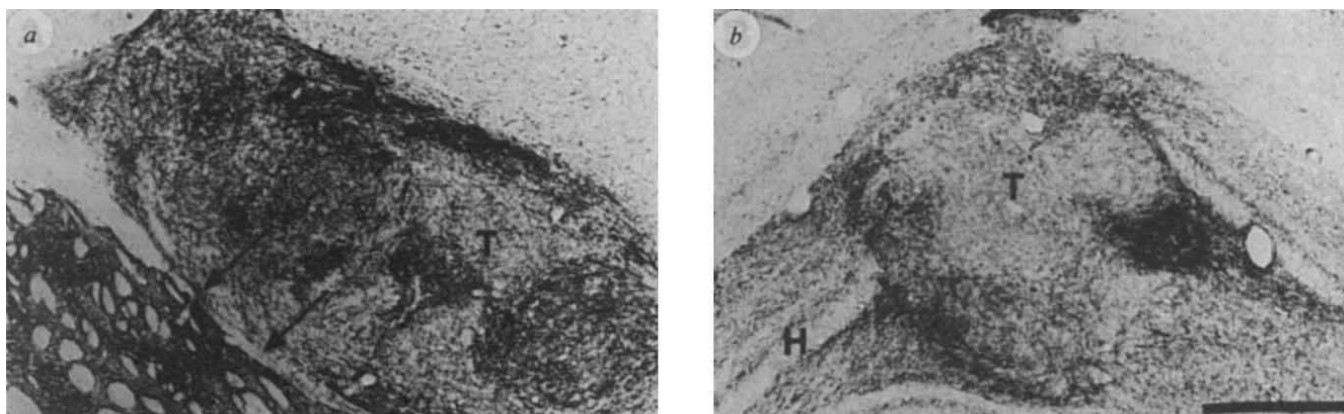
**Table 1** Effects of transplanted cholinergic and non-cholinergic embryonic brain tissue on the impaired cholinergic and noradrenergic system in the alcohol-damaged rat brain

|                                                     | ACh content<br>(pmol mg <sup>-1</sup> ww) | ACh release<br>(pmol mg <sup>-1</sup> ww) | Choline uptake<br>(pmol mg <sup>-1</sup> min <sup>-1</sup> ) | ChAT<br>(nmol <sup>-1</sup> mg h <sup>-1</sup> ) | NA content<br>(pmol mg <sup>-1</sup> ww <sup>-1</sup> ) |
|-----------------------------------------------------|-------------------------------------------|-------------------------------------------|--------------------------------------------------------------|--------------------------------------------------|---------------------------------------------------------|
| Non-alcohol treated controls                        |                                           |                                           |                                                              |                                                  |                                                         |
| Cortex                                              | 25.7 (0.5)                                | 10.2 (0.6)                                | 8.1 (0.3)                                                    | 47.7 (1.1)                                       | 1.4 (0.1)                                               |
| Hippocampus                                         | 28.8 (0.9)                                | 11.5 (0.6)                                | 13.4 (0.6)                                                   | 79.6 (1.8)                                       | 2.2 (0.1)                                               |
| Alcohol treated animals, preoperative               |                                           |                                           |                                                              |                                                  |                                                         |
| Cortex                                              | 14.6 (0.4)                                | 5.1 (0.4)                                 | 6.1 (0.2)                                                    | 36.7 (0.9)                                       | 0.8 (0.1)                                               |
| Hippocampus                                         | 15.8 (0.5)                                | 5.6 (0.3)                                 | 9.4 (0.4)                                                    | 55.7 (1.2)                                       | 1.4 (0.1)                                               |
| Cholinergic transplants into cortex                 |                                           |                                           |                                                              |                                                  |                                                         |
| Cortex                                              | 19.5 (0.4)***                             | 7.8 (0.6)**                               | 6.8 (0.3)                                                    | 41.1 (1.3)*                                      | 0.9 (0.1)                                               |
| Hippocampus                                         | 16.8 (0.5)                                | 6.0 (0.4)                                 | 9.6 (0.5)                                                    | 57.3 (1.3)                                       | 1.4 (0.1)                                               |
| Cholinergic transplants into hippocampus            |                                           |                                           |                                                              |                                                  |                                                         |
| Cortex                                              | 15.3 (0.4)                                | 5.5 (0.4)                                 | 6.3 (0.2)                                                    | 38.2 (0.9)                                       | 0.7 (0.1)                                               |
| Hippocampus                                         | 22.8 (0.5)***                             | 9.4 (0.5)***                              | 11.4 (0.5)*                                                  | 66.9 (0.8)***                                    | 1.5 (0.1)                                               |
| Cholinergic transplants into cortex and hippocampus |                                           |                                           |                                                              |                                                  |                                                         |
| Cortex                                              | 19.5 (0.3)***                             | 8.0 (0.6)**                               | 7.2 (0.3)*                                                   | 43.0 (0.7)***                                    | 0.9 (0.1)                                               |
| Hippocampus                                         | 23.8 (0.6)***                             | 9.8 (0.6)***                              | 11.8 (0.5)**                                                 | 67.9 (0.6)***                                    | 1.1 (0.1)                                               |

Data are mean values (s.e.m.);  $n = 5$  animals. Choline uptake and ChAT values are per mg protein; other values are for wet weight of tissue. Values of alcohol-treated animals differ significantly from those of non-treated controls ( $P < 0.01$ ). Significantly different from pre-operative values of alcohol-treated animals: \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . Increases in cholinergic parameters were also seen in the basal forebrain but not the pons-medulla (data not shown) in brains bearing cholinergic transplants. No significant changes of any kind were produced by non-cholinergic transplants.

**Methods.** The acetylcholine (ACh) content was measured by luminometry<sup>28</sup> in tissue after focused microwave irradiation; and ACh release, by challenging tissue minces with a depolarizing concentration (50 mM) of KCl applied in the luminometric assay buffer. The  $\text{Ca}^{2+}$  dependent release of ACh was measured over a 5-min period. Choline uptake in synaptosomes prepared on Percoll gradients<sup>29</sup> was measured essentially as in ref. 30, choline acetyltransferase (ChAT) by the method of Fonnum<sup>31</sup>, and noradrenaline (NA) by HPLC with an electron capture detector. In the transplanted animals tissue for assay was taken from the middle of the transplant.





**Fig. 2** *a*, Acetylcholinesterase (AChE) histochemistry of a cholinergic-rich transplant (T) of fetal cells in the parietal cortex, adjacent to the corpus striatum (boundary arrowed), three months after implantation. The transplant displays AChE reactivity, with patches more intensely stained. *b*, A non-cholinergic transplant (T) in the hippocampus (H), showing far less AChE reactivity, though with occasional intensely stained areas. Scale bar: 0.5 mm.

**Methods.** The basal forebrain region (cholinergic-rich) or hippocampus (non-cholinergic) were dissected from rat embryos at 15–16 days' gestation (13–16 mm crown–rump length) and cell suspensions prepared<sup>20</sup>. Implants of either cholinergic-rich or non-cholinergic cell suspensions were made at two sites in cortex, or two sites in the hippocampus, or at all four sites, bilaterally. The coordinates<sup>32</sup> were as follows: cortex, AP +3.2 mm, L  $\pm$ 3.5, V (from dura) –3.0, and AP –1.0, L  $\pm$ 5.0, V –2.0; hippocampus, AP –4.3, L  $\pm$ 2.2, V –3.4, and AP –3.3, L  $\pm$ 1.5, V –3.8. Three months later, at the completion of behavioural testing, brains were removed and fixed overnight by immersion in 10% formol-saline. After infiltrating with 15% sucrose in phosphate buffered saline (pH 7.3), brains were frozen and 30  $\mu$ m cryostat sections thaw-mounted onto slides. Sections were then washed thoroughly in distilled water and histochemically reacted for the presence of AChE<sup>33</sup>.

sign of recovery for six months after the end of alcohol treatment, mimicking the irreversibility of the memory loss seen in alcoholic Korsakoff psychosis<sup>4</sup>. The absence of diencephalic lesions in the alcohol-treated rats confirms other evidence<sup>4,23</sup> that these are due, not to alcohol ingestion as such, but to the thiamine deficiency often associated with it, and questions whether such lesions in the Korsakoff brain play any causal role in the cholinergic, noradrenergic or memory dysfunctions seen in this condition. Similarly, the persistence of the alcohol-induced deficit in NA content, after the recovery of memory function, suggests that this deficit is not responsible for the disturbance in memory. Reversal of the alcohol-induced memory loss by transplantation of cholinergic-rich, but not cholinergic-poor, brain tissue supports the hypothesis that memory loss is due to cholinergic degeneration of a kind seen also in the Alzheimer brain<sup>2,3,5</sup>.

The time-course of memory recovery in the transplanted animals (about nine weeks) is comparable to that observed<sup>20</sup> for the establishment of local neural connections between the implant and the host brain. Brain tissue transplants have been shown to reverse cognitive deficits caused by experimental lesions to the brain<sup>20,24</sup> or ageing<sup>25</sup>. Our data demonstrate that such transplants are also successful in an animal model where there is a known pathogenic agent—prolonged alcohol consumption—implicated in human disease, so lending weight to the possibility of using transplants to treat cognitive impairments in man. The additive effects of transplants into cortex and hippocampus suggest that the cholinergic innervation of the forebrain acts in a coordinated manner. Since the transplants were into areas far from the normal location of cholinergic cell bodies, it is unlikely that they were responsible for point-to-point transmission of neural information or for encoding the detailed content of specific memories. A more diffuse, paracrine<sup>26</sup> function is implied, concerned with some general aspect of storage or retrieval of memories. One possibility, already proposed as a function for the cholinergic innervation of the hippocampal system<sup>27</sup>, is the coordination over wide regions of brain of the timing of related neural messages.

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# A presynaptic action of glutamate at the cone output synapse

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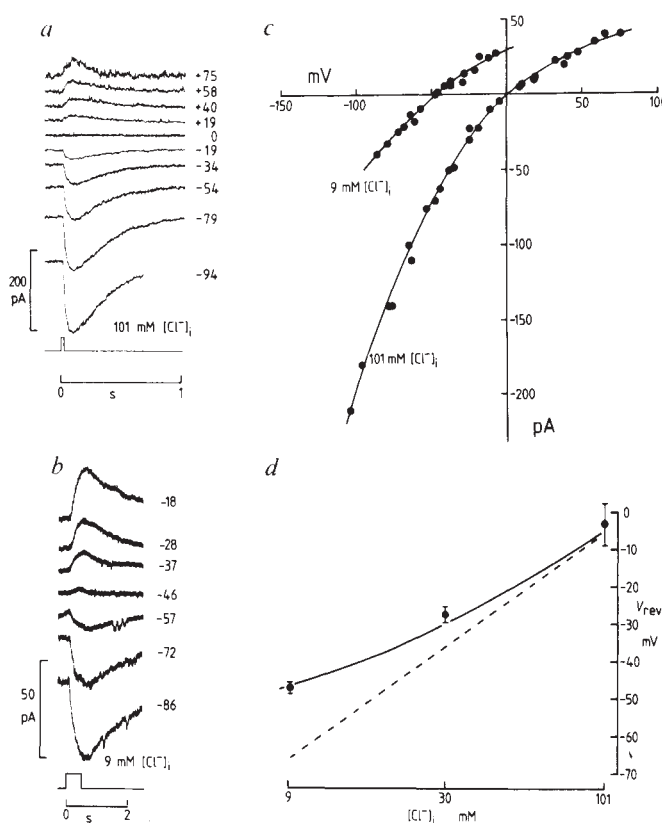
Neurotransmitter release from many central nervous system synapses is regulated by 'autoreceptors' at the synaptic terminal, which bind the released transmitter and alter release accordingly<sup>1</sup>. The photoreceptors of lower vertebrates are thought to use glutamate as a neurotransmitter<sup>2-4</sup>. Glutamate conveys the visual signal to postsynaptic bipolar and horizontal cells, but has been reported not to act on the photoreceptors themselves<sup>5</sup>. We show here that glutamate evokes a current, carried largely by chloride ions, in cones isolated from the tiger salamander retina. This response is localized to the synaptic terminal of the cone. Removing external sodium blocks this action of glutamate. These results suggest the existence of a positive feedback loop at the cone output synapse: over most of the light-response range, glutamate released by depolarization of the cone will cause further depolarization, increasing the gain of phototransduction. Glutamate released from rods may also polarize cones, modulating the gain of the cone output synapse. This system is surprisingly different from the autoreceptor systems for most other transmitters, which act in a negative feedback way<sup>1</sup>.

Iontophoretic application of L-glutamate increased the membrane conductance of isolated cones which were voltage-clamped using the whole-cell variant of the patch-clamp technique. With 101 mM chloride in the patch pipette, glutamate evoked an inward current at negative potentials, and an outward current at potentials above  $\sim 0$  mV (Fig. 1a). The dependence of the current on voltage showed inward rectification around the reversal potential (Fig. 1c). The inward current evoked at  $-40$  mV by glutamate iontophoresis, which under these conditions produces a near-maximum response (Fig. 3 legend), was typically 10–20 pA in amplitude, but sometimes as large as 60 pA (mean value  $18.5 \text{ pA} \pm 13.5 \text{ pA}$  (s.d.) in 25 cells).

When the patch pipette contained 9 or 30 mM chloride, the reversal potential of the current was more negative than with 101 mM chloride (Fig. 1b), indicating that a significant fraction of the current is carried by chloride ions. The dependence of the reversal potential on pipette chloride concentration (Fig. 1d) was weaker than is found for GABA ( $\gamma$ -aminobutyric acid)-evoked chloride currents in retinal neurones<sup>6,7</sup>, suggesting that the current evoked by glutamate in cones is less chloride-specific. The glutamate-evoked current was unaffected by bicuculline ( $100 \mu\text{M}$  on 10 cones) or strychnine ( $10 \mu\text{M}$  on 10 cones), at doses which greatly reduced or abolished chloride currents evoked in isolated retinal bipolar<sup>7</sup> or ganglion cells by iontophoresis of GABA or glycine. Glutamate usually gates non-specific cation channels in vertebrate neurones<sup>8</sup>, but glutamate-evoked chloride currents have been reported in invertebrate neurones<sup>9</sup> and muscle cells<sup>10</sup>.

Figure 2 shows the dependence of glutamate-evoked current on glutamate concentration. A significant fraction of the current was activated by  $1 \mu\text{M}$  glutamate. For the cell shown in Fig. 2a the dose-response curve (Fig. 2b) was roughly fitted by a Michaelis-Menten relation with  $K_m = 1.4 \mu\text{M}$ . In two other cones, Lineweaver-Burke plots gave  $K_m$  values of 2.6 and  $5.5 \mu\text{M}$  at  $-40$  mV. Kainate (1 or  $100 \mu\text{M}$ ) evoked currents similar to those induced by glutamate.

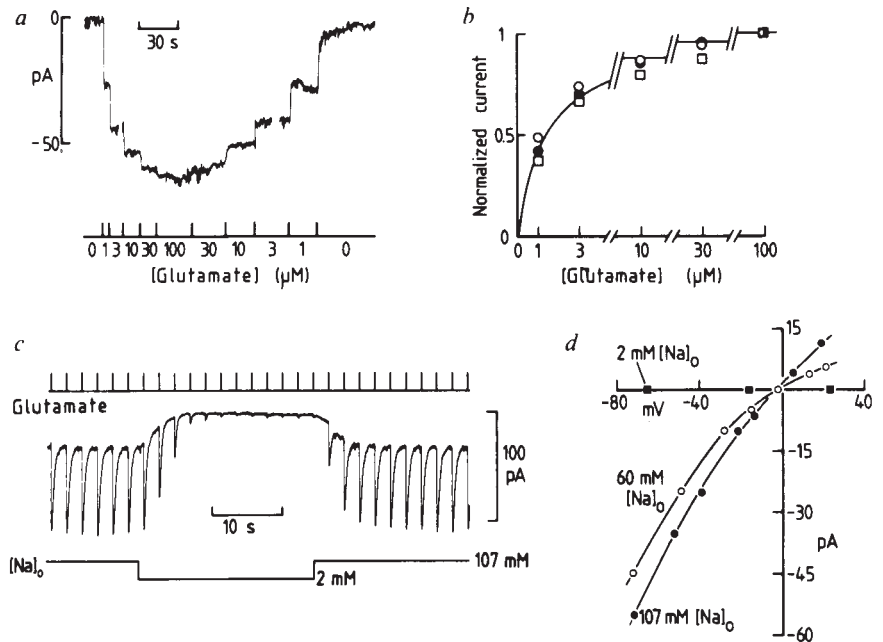
Removal of external sodium abolished the glutamate-evoked current (Fig. 2c). Lowering the external sodium concentration to 25, 50 or 60 mM (replacing it by choline) decreased the glutamate-induced current at all potentials, but did not detectably alter its reversal potential (Fig. 2d). Thus, although external sodium is needed for glutamate to evoke a current (perhaps



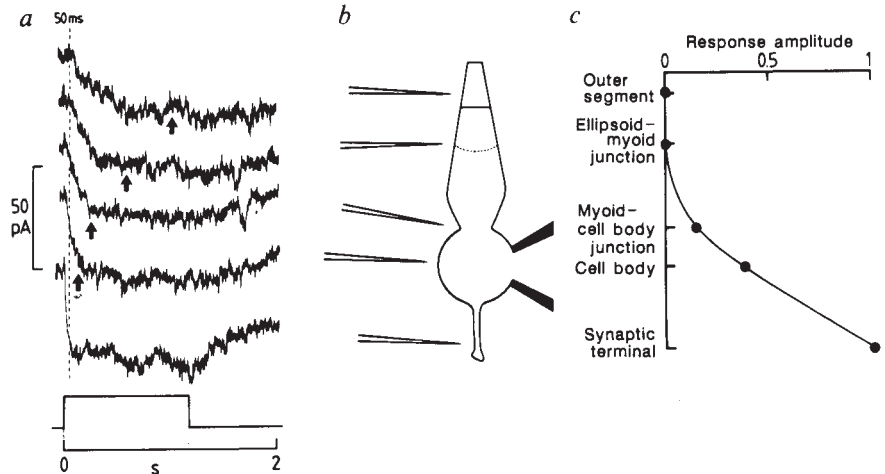
**Fig. 1** Voltage- and chloride-dependence of the glutamate-induced current in isolated cones. *a*, Changes in membrane current of a voltage-clamped cone in response to iontophoresis of glutamate. Membrane potential in mV shown by each trace. The external solution was barium-Ringer's; the patch pipette contained 101 mM  $[\text{Cl}^-]_i$  (see below). This example was half of a double cone: similar results were obtained from isolated single cones. *b*, Glutamate-evoked currents in a double cone (in barium-Ringer's) studied with 9 mM  $[\text{Cl}^-]_i$  in the patch pipette. *c*, Peak glutamate-induced currents (ordinate) as a function of membrane potential (abscissa) for the experiments of *a* and *b*. *d*, Reversal potential of the glutamate-induced current (ordinate) in barium-Ringer's as a function of pipette chloride concentration ( $[\text{Cl}^-]_i$ , abscissa). Bars show s.d. Four, six and ten cells studied for 9, 30 and 101 mM  $[\text{Cl}^-]_i$ . Data do not fit the Nernst prediction for a chloride-specific channel (dashed line), suggesting that glutamate opens relatively non-specific channels: smooth curve is the Goldman-Hodgkin-Katz equation for a channel with permeability ratio  $P_{\text{acetate}}/P_{\text{Cl}} = 0.1$ . **Methods.** Cones were isolated from the tiger salamander retina by papain dissociation<sup>20</sup>. Patch pipettes used for whole-cell recording contained (for 101 mM  $[\text{Cl}^-]_i$  in the pipette): 80 mM KCl; 15 mM potassium acetate; 5 mM NaCl; 5 mM HEPES; 7 mM  $\text{MgCl}_2$ ; 5 mM  $\text{Na}_2\text{ATP}$ ; 1 mM  $\text{CaCl}_2$ ; 5 mM  $\text{K}_2\text{EGTA}$ ; pH adjusted to 7.0 with KOH. For 30 mM  $[\text{Cl}^-]_i$ , 71 of the 80 mM KCl was replaced by potassium acetate. For 9 mM  $[\text{Cl}^-]_i$ , all the KCl was replaced by potassium acetate, and 6 mM  $\text{MgCl}_2$  was replaced by  $\text{Mg}(\text{acetate})_2$ . Tip potentials were corrected for<sup>21</sup>. Normal external Ringer's contained: 105 mM NaCl; 2.5 mM KCl; 3 mM  $\text{CaCl}_2$ ; 0.5 mM  $\text{MgCl}_2$ ; 15 mM glucose; 5 mM HEPES; pH adjusted to 7.3 with NaOH. Experiments were often carried out in barium-Ringer's (normal Ringer's plus 6 mM  $\text{BaCl}_2$ ): barium increased the cone resistance and decreased the current noise at positive potentials, allowing glutamate-induced currents to be studied over a larger potential range. With 101 mM  $[\text{Cl}^-]_i$  in the patch pipette, barium had no effect on the magnitude of the glutamate-induced current in the physiological potential range. Glutamate was applied by addition to the external solution, or by iontophoresis from pipettes filled with 1 M sodium glutamate (at pH 8, resistance 30 M $\Omega$  in Ringer's, a holding current of +30 nA was switched to  $-40$  nA for ejection). Results obtained with perfused glutamate (Fig. 2) were similar to those obtained with iontophoresis, ruling out the possibility that iontophoresis evokes a current by generating a local pH change.



**Fig. 2** Glutamate- and sodium-dependence of the glutamate-evoked current. *a*, Current evoked in a single cone voltage-clamped to  $-42$  mV, by different concentrations of superfused glutamate applied in barium Ringer's. *b*, Normalized dose-response curve obtained from the data in *a* (●) and from similar experiments on the same cell at  $-62$  mV (○) and at  $+28$  mV (□). The currents evoked at these potentials by  $100 \mu\text{M}$  glutamate were, respectively,  $-63$ ,  $-93$  and  $+24$  pA. The curve through the points is a Michaelis-Menten relation with  $K_m = 1.4 \mu\text{M}$  (as obtained from a Lineweaver-Burke plot of the data for  $-42$  mV). *c*, The glutamate-induced current is sodium-dependent. While glutamate was repeatedly applied by iontophoresis (top trace) to an isolated double cone in barium Ringer's, the sodium concentration in the superfusion solution was reduced from 107 to 2 mM (bottom trace, sodium replaced by choline). The inward membrane current (middle trace) evoked by glutamate at  $-50$  mV was greatly reduced by reducing  $[\text{Na}]_o$ . The change of external sodium takes about 8 seconds to occur. Removal of external sodium did not result in glutamate evoking an outward current at  $-50$  mV, as might be expected if glutamate opened channels permeable to sodium and potassium ions. *d*, Current-voltage relations for the current induced by glutamate iontophoresis in a single cone in barium Ringer's containing 107, 60 or 2 mM  $[\text{Na}^+]$ . No current was detectable at any potential in 2 mM  $[\text{Na}^+]$ . Sodium removal decreases the current without detectably changing its reversal potential (experiment performed on 11 cells). Pipettes contained 101 mM  $[\text{Cl}^-]$  for all parts of this figure.



**Fig. 3** The glutamate-induced current is restricted to the synaptic terminal of the cone. *a*, Responses to glutamate applied by iontophoresis  $5 \mu\text{m}$  away from a whole-cell clamped cone at the positions shown in *b*. Holding potential  $-72$  mV. Iontophoresis timing shown as bottom trace. External solution was normal Ringer's; the pipette contained 101 mM  $[\text{Cl}^-]$ . When glutamate is applied at the synaptic terminal the response is fast in onset. As the iontophoresis electrode is moved to other cellular locations, more distant from the terminal, the response becomes slower because of the time needed for glutamate to diffuse to the terminal. The arrows by the traces show an estimate, for each position of the iontophoretic electrode, of the time at which the glutamate concentration at the synaptic terminal will reach one third of its maximum value (calculated as  $x^2/2D$ , where  $x$  is the distance between the electrode tip and the terminal, and  $D$ , the diffusion coefficient for glutamate, was taken as  $10^{-9} \text{ m}^2 \text{ s}^{-1}$ ). Even with the electrode positioned at the cone outer segment, the glutamate-induced current eventually reaches two thirds of the amplitude of that produced by iontophoresis at the synaptic terminal. We attribute this to the high concentration of glutamate produced by iontophoresis: from earlier experiments on glial cells<sup>11</sup>, we estimate the steady-state glutamate concentration at a distance of  $50 \mu\text{m}$  (the cone length) from the iontophoretic electrode to be about  $10 \mu\text{M}$ , a concentration which generates an almost maximum response in the cone (Fig. 2*b*). *c*, Normalized plot of the localization of the glutamate response, obtained by measuring the currents in *a*, 50 ms after the start of iontophoresis (vertical dashed line in *a*). At this time glutamate will have diffused only about  $10 \mu\text{m}$  from the iontophoretic electrode (that is,  $\sim 1.5$  times the length of the synaptic terminal). This plot indicates localization of the response to the synaptic terminal. Cones which had lost their synaptic terminals in the cell isolation procedure did not respond to glutamate.

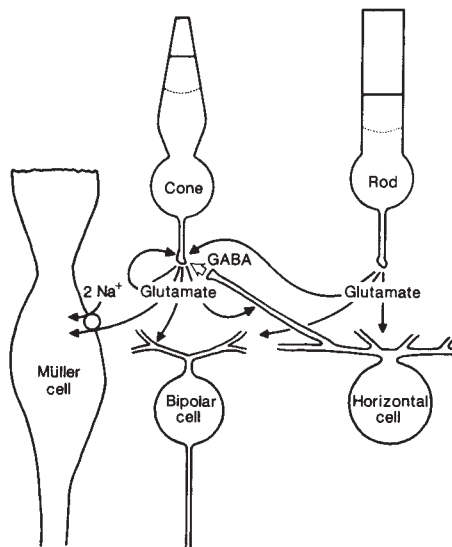


because sodium ions are required for glutamate to bind to its receptor, or to maintain a low calcium concentration in the cone synaptic terminal via sodium-calcium exchange), sodium does not appear to pass through the ion channels opened by glutamate.

The spatial localization of the glutamate-activated current was mapped by iontophoresis of glutamate at various positions around the cone (Fig. 3). The current was largest and fastest in onset when glutamate was applied near the synaptic terminal,

demonstrating that the response is generated in the terminal. At other locations the response was smaller, and notably slower in onset, presumably due to the time needed for glutamate to diffuse to the synaptic terminal. Consistent with this, cones which had lost their synaptic terminals in the cell-isolation procedure did not respond to glutamate.

Figure 4 summarizes the sites of action of glutamate released from the cone output synapse. As well as gating ion channels on postsynaptic bipolar and horizontal cells, and being taken



**Fig. 4** The actions of glutamate released from cones and rods. Glutamate gates ion channels in postsynaptic bipolar and horizontal cells, allowing passage of the visual signal to these second-order neurones. Bipolar cells pass the visual signal to the output (ganglion) cells of the retina; horizontal cells mediate lateral inhibition in the outer retina. The synaptic action of glutamate is terminated by sodium-dependent uptake<sup>11</sup> into glial (Müller) cells (and possibly into cones and rods) and by diffusion away in the extracellular space. Also, glutamate released from cones can act on the receptors in the cone synaptic terminal. Although the exact distance between the glutamate release sites and glutamate receptors on the synaptic terminal is not known, glutamate diffusion (in the extracellular space) all the way from the bottom of the synaptic terminal to the cone cell body will take only about 50 ms — less than the time needed for the cone to respond to increases or decreases of illumination<sup>12</sup>. Cones also receive an inhibitory input: a synapse releasing GABA (open arrow) from horizontal cells<sup>6,15</sup>. Histological work<sup>13</sup> suggests that glutamate released from rods may also polarize cones, modulating the gain of the output synapse of the cone (if this has a voltage-dependence similar to that found for rods<sup>14</sup>, where depolarization increases the synaptic gain).

up into glial cells<sup>11</sup>, glutamate will also evoke a current in the cone it is released from (the magnitude of which will depend on how close the cone glutamate receptors are to the sites of glutamate release). The chloride concentration in (turtle) cones has been estimated<sup>6</sup> to be between 12 and 24 mM, suggesting a reversal potential for the glutamate-evoked current *in vivo* between  $-34$  and  $-43$  mV (from Fig. 1d). Thus, over most or all of the cone light-response range<sup>12</sup> ( $-40$  to  $-65$  mV) glutamate will evoke an inward current in the cone and, when a stimulus (removal of light) depolarizes a cone, the feedback action of the glutamate released will further depolarize the cone. As a result, the gain for the conversion of changes in light intensity into changes of glutamate release will be increased. This can be seen as follows. If a change of light intensity generates a current change,  $\Delta I_{\text{light}}$ , in the cone outer segment, the resulting change of cone voltage,  $\Delta V$ , will be determined by the cone resistance,  $R$ , and by the change in the current,  $\Delta I_{\text{glu}}$ , evoked by glutamate binding to autoreceptors in the cone synaptic terminal:

$$\Delta V = R(\Delta I_{\text{light}} + \Delta I_{\text{glu}}) \quad (1)$$

(note that vertebrate photoreceptors do not generate action potentials, but code light intensity with graded potential changes). Assuming, for simplicity, that for small changes of light intensity the change in glutamate-evoked current is proportional to the local change in glutamate concentration, which in turn is assumed to be proportional to the membrane potential change (so that  $\Delta I_{\text{glu}} = k\Delta V$  with  $k$  a constant), equation (1) becomes:

$$\Delta V = R\Delta I_{\text{light}}/(1 - kR) \quad (2)$$

Thus, for  $kR < 1$  (that is, feedback too weak to produce a regenerative voltage change), the changes in cone voltage and glutamate release are increased by a factor of  $1/(1 - kR)$  relative to the situation with no autoreceptors ( $k = 0$ ). This gain increase could be significant, because the glutamate-gated conductance in the cone (for example 1 nS for the cell of Fig. 1b) can be comparable to the conductance modulated by light<sup>12</sup> (0.94 nS).

The glutamate receptors on the synaptic terminal of the cone may also respond to glutamate released from rods: there is histological evidence for a synapse from rods to cones<sup>13</sup>. If the output synapse of the cone has a voltage-dependent gain (as for rods<sup>14</sup>), this would lead to modulation of the cone synapse gain by the rods in the mesopic range of light intensities.

Previous work<sup>5</sup> has shown that when glutamate is perfused onto the intact retina cones are not polarized as would be expected from Fig. 4. This may result (in the dark) from the proximity of the dark potential to the reversal potential for the

glutamate-gated current, and (in the light) from the applied glutamate depolarizing horizontal cells<sup>2</sup>, which send an inhibitory synapse to cones (Fig. 4), hyperpolarizing them<sup>6,15</sup>. For bulk perfusion of glutamate the horizontal cells will undergo a large voltage change, and the resulting inhibitory action on cones could outweigh the direct depolarizing action of glutamate. For spatially restricted illumination changes, however, the horizontal cells undergo little voltage change as a result of changes in transmitter release from one or a few cones (a small fraction of the cones projecting to each horizontal cell), and the positive feedback loop we have characterized will not be outweighed by inhibitory horizontal cell input.

As glutamate receptors on synaptic terminals have been suggested to exist in the vertebrate cerebellum<sup>16</sup>, hippocampus<sup>17</sup> and olfactory cortex<sup>18</sup> (and at the crustacean neuromuscular junction<sup>19</sup>), the mechanism we have characterized may be of widespread importance in the central nervous system. With one exception (the  $\beta$  receptors on noradrenergic terminals), the autoreceptor systems for various neurotransmitters characterized to date have been found to operate in a negative feedback way<sup>1</sup>. The existence of an autoreceptor-mediated positive feedback loop for a fast excitatory transmitter like glutamate is surprising, as it provides a mechanism which may, in the case of malfunction, lead to runaway neuronal excitation.

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**Note added in proof:** A. Kaneko and M. Tachibana (*Invest. Ophthalmol. Suppl.* **28**, 50 (1987)) have reported a glutamate-evoked current in turtle photoreceptors.

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# The neurotrophic factor neuroleukin is 90% homologous with phosphohexose isomerase

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Neuroleukin (NLK) is a protein of relative molecular mass ( $M_r$ ) 56,000 (56K) secreted by denervated rat muscle<sup>1</sup> and found in large amounts in muscle, brain, heart and kidneys<sup>2</sup>. The protein is a neurotrophic factor for spinal and sensory neurons<sup>2</sup> and a lymphokine product of lectin-stimulated T-cells<sup>3</sup>. It also induces immunoglobulin secretion by human mononuclear cells<sup>3</sup>. Molecular clones of NLK have been expressed in monkey COS cells and the product was shown to have the same biological and biochemical properties as the extracted protein. NLK is abundant in muscle, brain and kidney, but is active at concentrations of  $10^{-9}$  to  $10^{-11}$  M, similar to those for other polypeptide factors. We have cloned the gene for pig muscle phosphohexose isomerase (PHI) (EC 5.3.1.9) which catalyses the conversion of glucose-6-phosphate to fructose-6-phosphate, an obligatory step in glycolysis, and determined its amino-acid sequence. Surprisingly, it is 90% homologous to the sequence of mouse neuroleukin.

Several observations indicate that the protein sequence translated from our cloned putative PHI complementary DNA is that of mature pig PHI (Fig. 1). The C-terminus end has the same five residues as those determined by Achari *et al.*<sup>4</sup> by carboxypeptidase digestion. The first AUG codon, which we consider to be the start codon, is embedded in a canonical sequence for a eukaryotic translation initiation site (CC<sub>6</sub>CCAUGG)<sup>5</sup>. Pig PHI is resistant to Edman degradation, suggesting that the terminal  $\alpha$ -amino group may be blocked. Pronase digestion showed this to be due to an N-terminal acetylalanine residue and this is consistent with the occurrence of alanine in position 1 and 2, but not elsewhere within the first 60 amino-acids of our derived sequence. Lastly, the amino-acid composition determined by Achari *et al.*<sup>4</sup> fits almost perfectly with the sequence translated in Fig. 1.

Comparison of the derived PHI amino-acid sequence with sequences in the NBRF (National Biomedical Research Foundation) data bank revealed its homology with mouse neuroleukin (Fig. 1). The two sequences have 90% homology, and both contain 558 residues and can be aligned without any requirement for insertions or deletions. The differences are mainly conservative replacements and probably reflect species and organ specificity. Of the four cysteine residues present in mouse NLK, only three are in pig PHI (residues 133, 333 and 404), the cysteine in position 330 being replaced by phenylalanine. This observation is of limited importance, however, as previous studies have shown that pig PHI contains no disulphide bonds, and that at least two of its sulphhydryl groups are unimportant for enzyme activity. Similarly no disulphide bond is involved in NLK activity. Three potential N-dependent glycosylation sites are found at positions 105, 129, and 249 in pig PHI and at positions 39, 91, and 129 in mouse NLK but neither molecule has been shown to be glycosylated. In contrast to the coding sequences, the untranslated RNA sequences at the 3' end of the pig PHI and mouse NLK RNAs are strikingly divergent.

Tissues with the highest NLK concentrations are also those

|                                                                                     |      |     |     |     |     |     |     |     |     |     |     |      |
|-------------------------------------------------------------------------------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| TTTTTTTTTTTGTAGTTTCCAGCGGCTTTCGCC                                                   | ATG  | GCT | GCA | CTC | ACC | CAG | AAC | CCG | CAG | TTC | AAG | 69   |
|                                                                                     | Met  | Ala | Ala | Leu | Thr | Gln | Asn | Pro | Gln | Phe | Lys | pPHI |
|                                                                                     |      |     |     |     |     |     | Arg |     |     |     | Gln | mNLK |
| AAG CTG CAG ACC TGG TAC CAC GAG CAC CGC TCT GAC CTC AAC TTG CGC CGC CTT TTC GAA     | 129  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Lys Leu Gln Thr Trp Tyr His Glu His Arg Ser Asp Leu Asn Leu Arg Arg Leu Phe Glu     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - - - Leu Glu - - His Arg Ala Asn Ser Ala Asn - - Lys - - - Glu - - -               |      |     |     |     |     |     |     |     |     |     |     |      |
| GGG GAC AAG GAC CGC TTC AAC CAC TTC AGC TTG AAC CTC AAC ACC AAC CAT GGC CGT ATT     | 189  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Gly Asp Lys Asp Arg Phe Asn His Phe Ser Leu Asn Leu Asn Thr Asn His Gly Arg Ile     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| Ala - - - Pro Glu - - - - - Asn - - - - - - - - - - - His - - -                     |      |     |     |     |     |     |     |     |     |     |     |      |
| CTG TTG GAT TAC TCC AAG AAT CTT GTG ACG GAG GCT GTG ATG CAG ATG CTG GTG GAC CTG     | 249  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Leu Leu Asp Tyr Ser Lys Asn Leu Val Thr Glu Ala Val Met Gln Met Leu Val Asp Leu     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - Val - - - - - - - - - - - Asn Lys Glu - - - - - - - - - - - Glu - - -             |      |     |     |     |     |     |     |     |     |     |     |      |
| GCC AAG TCC AGG GGT GTS GAG GCT GCC CGG GAG CGC ATG TTC AAT GGT GAG AAG ATC AAC     | 309  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Ala Lys Ser Arg Gly Val Glu Ala Ala Arg Glu Arg Met Phe Asn Gly Glu Lys Ile Asn     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - - - - - Asp Asn - - - - - Ser - - - - -                                           |      |     |     |     |     |     |     |     |     |     |     |      |
| TTC ACC GAG GAT CGG GCA GTG TTG CAC GTG GCC CTG AGG AAC CGG TCA ACA ACA CCC ATT     | 369  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Phe Thr Glu Asp Arg Ala Val Leu His Val Ala Leu Arg Asn Arg Ser Arg Thr Pro Ile     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| Tyr - - - - - - - - - - - Asn Lys Glu - - - - - - - - - - -                         |      |     |     |     |     |     |     |     |     |     |     |      |
| CTA GTC GAG CGC AAG GAT GTS ATG CCA GAG GTC AAC AGG GTC CTG GAG AAG ATG AAG TCT     | 429  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Leu Val Asp Asp Gly Lys Asp Val Met Pro Glu Val Asn Arg Val Leu Glu Lys Met Lys Ser |      |     |     |     |     |     |     |     |     |     |     |      |
| - - - - - I - - - - -                                                               |      |     |     |     |     |     |     |     |     |     |     |      |
| Lys - - - - - - - - - - - Asp - - - - - Asn - - - - -                               | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| TTC TGC AAG CGT GTC CGG AGT GCC GAA TGG AAA GGG TAC TCG GGC AAG TCC ATC ACG GAC     | 489  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Phe Cys Lys Arg Val Arg Ser Gly Glu Thr Lys Gly Tyr Ser Gly Lys Ser Ile Thr Asp     |      |     |     |     |     |     |     |     |     |     |     |      |
| - - - Gln - - - - - CB-I - - - - - Asp - - - - - Thr - - - - - - - - - - -          | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| GTC ATC AAC ATC GGC ATC GGC GGC TCT GAC CTG GGA CCC CTC ATG GTG ACG GAA GGC CTC     | 549  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Val Ile Asn Ile Gly Ile Gly Gly Ser Asp Leu Gly Pro Leu Met Val Thr Glu Ala Leu     |      |     |     |     |     |     |     |     |     |     |     |      |
| Ile - - - - - I - - - - -                                                           | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| AAG CCG TAC TCT GCA GAA GGC CCC CGG GTC TGG TTC GTC TCC AAC ATT GAT GGC ACC CAC     | 609  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Lys Pro Tyr Ser Ala Glu Gly Pro Arg Val Trp Phe Val Ser Asn Ile Asp Gly Thr His     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - - - - - Lys Gly - - - - - Arg - - - - - Thr - - - - - - - - - - -                 |      |     |     |     |     |     |     |     |     |     |     |      |
| ATT GCC AAA ACG CTG GCC ACC CTG AAC CCC GAG TCG TCC CTG TTC ATC ATT GCC TCC AAG     | 669  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Ile Ala Lys Thr Leu Ala Thr Leu Asn Pro Gln Asn Met Phe Glu Phe Thr Thr Thr Lys     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - - - Ser - - - Ser - - - Thr - - - - -                                             |      |     |     |     |     |     |     |     |     |     |     |      |
| ACC TTC ACC ACC CAG GAG ACC ATC ACA AAT GCA GAG ACG GCG AAG GAG TGG TTT CTT CAG     | 729  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Thr Phe Thr Thr Thr Gln Glu Thr Ile Thr Asn Ala Glu Thr Ala Lys Glu Thr Phe Leu Gln | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - - - Thr - - - - - - - - - - - Ile - - - - - - - - - - - Glu - - -                 |      |     |     |     |     |     |     |     |     |     |     |      |
| TCA GCC AAG GAT CCC TCT GCA GTC GCA AAG CAC TTT GTC GCC CTG TCT ACC AAC ACT ACC     | 789  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Ser Ala Lys Asp Pro Ser Ala Val Ala Lys His Phe Val Ala Leu Ser Thr Asn Thr Thr     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - - - Ala - - - - - - - - - - - - - - - - - Ala - - -                               |      |     |     |     |     |     |     |     |     |     |     |      |
| AAA GTG AAG GAA TTT GGA ATT GAT CCT CAA AAC ATG TTC GAG TTC TGG GAT TGG GTG GGA     | 849  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Lys Val Lys Glu Phe Gly Ile Asp Pro Gln Asn Met Phe Glu Phe Thr Asp Thr Val Gly     |      |     |     |     |     |     |     |     |     |     |     |      |
| - - - I - - - - - S-I - - - - - I - - - - -                                         | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| GGC CGC TAC TCG CTC TGG TCA GCC ATC GGA CTC TCC ATT GCC CTG CAC GTG GGG TTT GAC     | 909  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Gly Arg Tyr Ser Leu Trp Ser Ala Ile Gly Leu Ser Ile Ala Leu His Val Gly Phe Asp     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - - - - - CB-II - - - - -                                                           |      |     |     |     |     |     |     |     |     |     |     |      |
| AAC TTC GAG CAG CTG CTC TCG GGG GCT CAC TGG ATG GAC CAG CAC TTC CGC ACG ACA CCC     | 969  |     |     |     |     |     |     |     |     |     |     | pPHI |
| Asn Phe Glu Gln Leu Leu Ser Gly Ala His Trp Met Asp Gln His Phe Arg Thr Thr Pro     | mNLK |     |     |     |     |     |     |     |     |     |     |      |
| - - - - - I - - - - -                                                               |      |     |     |     |     |     |     |     |     |     |     |      |
| His - - - - - - - - - - - - - - - - - Leu Lys - - -                                 |      |     |     |     |     |     |     |     |     |     |     |      |

**Fig. 1** DNA and translated amino-acid sequence of pig PHI (pPHI) compared to the mouse NLK (mNLK) sequence. Locations of sequenced peptides of pig PHI are underlined: CB-I to CB-V are cyanogen bromide fragments (Achari *et al.*<sup>4</sup>), S-I and S-II are V8 staphylococcus protease fragments of our pig PHI preparation, CP is the C-terminus sequence (Achari *et al.*<sup>4</sup>). Protein sequences determined chemically or translated from the cDNA clone are in complete agreement. Positions that vary between pPHI and mNLK are indicated in italics for conservative changes and in dark letters for full changes. Alignment starts from the initiation ATG codons.

**Methods.** Pig PHI was purified as described<sup>8</sup> yielding 63 mg of enzyme from 390 g of tissue. After digestion with V8 protease, and separation by reversed-phase HPLC, two fragments were sequenced by gas phase sequencer analysis. Anti-PHI rabbit polyclonal antibody was obtained by three immunizations at two week intervals using 116  $\mu$ g of protein emulsified in complete Freund's adjuvant. Mono-

with very high glycolytic activity where PHI is known to be important. PHI is, however, also present in serum and an increase in its activity in serum has been proposed as a marker in various diseases<sup>6</sup>. Release of PHI from muscle cells could create a concentration gradient and provide physiological significance to the observation that NLK promotes the survival of embryonic spinal neurons in cultures that probably include skeletal motor neurons.

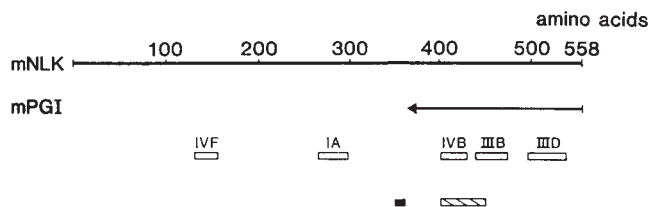
Neuroleukin was also shown to be a lymphokine product of lectin-stimulated T-cells. It is not surprising that T-cell stimulation by three different lectins increased the level of PHI messenger RNA, probably because of increased energy requirement, but the lymphokine effect upon Ig release by B-cells is less expected. Possibly NLK itself acts in a lectin-like fashion and NLK has been shown to bind to a surface component of

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Recently, the gp120 envelope glycoproteins of human immunodeficiency virus 1 (HIV-1) and simian immunodeficiency virus (SIV) were found to inhibit neuron growth in the presence of NLK<sup>7</sup>. Sequence homology has been identified between NLK (residues 403-447) and a conserved domain of HIV-1 gp120 (residues 238-282). It was postulated that this might be important for this inhibitory property of gp120, and that interactions between gp120 and NLK might play a role in the pathogenesis of AIDS (acquired immune deficiency syndrome)-related dementia.

We screened a mouse cDNA library with the PGI 1 gene of *Saccharomyces cerevisiae*,<sup>4</sup> and isolated a recombinant containing a 3.7 kilobase (kb) insert which we sub-cloned into M13 for DNA sequence analysis. Comparison with the GenBank sequence data bank revealed a 100% sequence identity between the 759 nucleotides at the 3' end of the mouse PGI which we sequenced and bases 1,164–1,922 of mouse neuroleukin. The sequence of mouse PGI also shows 87% homology with the coding region of human neuroleukin; however, there is a significant loss of homology (down to 60%) in the 3' non-coding region. We have sequenced nearly 70% of the yeast gene (unpublished data); it has a homology of 61% with both mouse PGI and mouse neuroleukin. Further confirmation that neuroleukin and PGI are identical (or closely related) comes from a comparison of published peptide sequence data obtained from





**Fig. 1** The extent of the deduced amino-acid sequence of mouse neuroleukin (mNLK) and mouse glucose-6-phosphate isomerase (mPGI). Open boxes show the positions of the sequenced peptides obtained by cyanogen bromide cleavage of porcine PGI (IVF, IA, IVB, IIB and IID)<sup>5</sup>; the hatched box shows the position of the amino acid sequence of a conserved region of the gp120 envelope protein of HIV that has partial homology to neuroleukin<sup>9</sup>; the filled box shows the position of the amino acid sequence of the T36 peptide used by Gurney *et al.*<sup>1</sup> to synthesize the oligonucleotide probe used to screen a mouse cDNA library.

**Methods.** A  $\lambda$ gt10 cDNA library from cultured mouse EK cells obtained from F. Pourier (NIMR) was screened with a 2.0 kb *Eco*RI fragment containing the gene for yeast PGI (PGI 1)<sup>4</sup>. One clone, designated pgi 6.1, contained a 3.7 kb insert which was subcloned into M13 vectors for sequence analysis of both strands using the dideoxy-chain termination method<sup>10</sup>.

cyanogen bromide cleavage of purified and crystallized porcine PGI<sup>5</sup>. The amino acid sequence of all five of the cyanogen bromide fragments described can be aligned with the deduced amino acid sequence of mouse neuroleukin, and three can be aligned with the deduced amino acid sequence of the 3' end of mouse PGI (Fig 1).

Our strategy for isolating the cDNA of mouse PGI relied on the known conservation of protein sequence between glycolytic enzymes<sup>6</sup> and predicted that the yeast PGI gene would be a specific, selective probe. A DNA encoding mouse neuroleukin has previously been isolated by purifying neuroleukin from mouse salivary glands, and using the amino acid sequence of a peptide (T36) derived from tryptic digestion to synthesize an oligonucleotide probe for screening a mouse cDNA library<sup>1</sup>. Although we have not yet sequenced enough of our PGI clone to determine whether the T36-encoding sequence is present in mouse PGI, we would predict that it is as T36 shows 67% homology to yeast PGI. It therefore seems probable that the protein actually isolated from mouse salivary glands<sup>1</sup> was PGI, perhaps in association with the neurotrophic factor, neuroleukin. It seems unlikely that neuroleukin is a contaminant of PGI, however, because Gurney *et al.* were able to show that monkey COS-1 cells transfected with the mouse neuroleukin cDNA synthesized a product with all the properties of neuroleukin, the activity of which could be neutralized by a monoclonal antibody directed against neuroleukin<sup>1</sup>. Transfected and control cells would both contain PGI as it is an ubiquitous enzyme. Therefore, unless mouse, but not monkey, PGI is secreted, it appears that the neurotrophic properties of the protein are highly species-specific. It will be interesting to determine whether neuroleukin has PGI activity, conversely whether PGI has the neurotrophic and immunomodulating properties ascribed to neuroleukin and lastly whether the two proteins are identical or closely related products processed from the same gene.

Neuroleukin has been associated with motor neurone disease<sup>7</sup> and, more recently, with the neuropathology sometimes found with AIDS<sup>8</sup>. The latter association is particularly intriguing as it is based on a partial sequence homology of neuroleukin (and hence PGI) with a conserved domain within the gp120 envelope glycoprotein of human immunodeficiency virus (HIV-1)<sup>9</sup>. The gp120 protein has been shown to inhibit the biological response of cultured neurones to neuroleukin but not to nerve growth factor<sup>9</sup>. A 19-amino-acid synthetic peptide containing the HIV sequence homologous to neuroleukin also blocked the action of neuroleukin<sup>8</sup>. It has been suggested that this sequence might play a role in the neuropathogenicity of HIV by gp120 and its

breakdown products competing with neuroleukin for binding to certain neurones, hence shortening their survival and disrupting normal neuronal function<sup>8,9</sup>. Whether this homology does have any such biological or pathological significance deserves further investigation.

We thank Mr J. Keyte and Dr A. Hawkins for synthesis of oligonucleotide primers and Dr A. Aguilera for the yeast PGI gene. The Wellcome Trust is thanked for continued support.

**Note added in proof:** We have now sequenced past the T36-encoding sequence and confirmed its presence in mouse PGI.

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**GURNEY REPLIES**—Faik *et al.*<sup>1</sup> and Chaput *et al.*<sup>2</sup> have shown that the polypeptide that we had previously described as having properties that suggested the name neuroleukin<sup>3</sup> is, in fact, glucophosphoisomerase (GPI). These authors isolated and sequenced complementary DNA clones of GPI and discovered that the sequences showed remarkable homology to that of mouse neuroleukin, which we had published at a time when the GPI sequences were not available in either the Genbank or the National Biomedical Research Foundation databanks. We find that a cDNA encoding human neuroleukin<sup>5</sup> maps to the same region as GPI and the genes for the lipoproteins C1, C2 and E on the long arm of human chromosome 19. The pattern of restriction fragments in human DNA suggests a single gene and we find no evidence for alternative transcripts. We have been able to confirm that both the mouse and human neuroleukin cDNAs express GPI enzymatic activity when transfected into monkey COS cells. GPI is secreted by the transfected cells and levels of GPI in the culture supernatant reach 20-30% of that in the cell lysate.

We previously reported two very different biological activities for recombinant mouse neuroleukin: first, selective stimulation of spinal and sensory neuron survival in culture with no effect on ciliary or sympathetic neurons; and second, triggering of polyclonal immunoglobulin secretion by cultured human peripheral blood mononuclear cells<sup>3,4</sup>. These experiments were performed using supernatants from cultured COS cells transfected with the neuroleukin cDNA. These biological effects of recombinant neuroleukin were blocked by two monoclonal antibodies (designated B130.6 and B109.9) that recognize purified neuroleukin protein, and also by a rabbit antiserum to neuroleukin but not by the pre-immune serum. COS cells mock-transfected with expression vector DNA only did not release any factor with neuroleukin activity. We have now re-examined the effects of the monoclonal antibodies and the rabbit antiserum in light of the identity between neuroleukin and GPI. The monoclonal antibodies and the rabbit immune serum, but not the pre-immune serum, react in an enzyme-linked immunosorbent assay (ELISA) with highly purified GPI obtained from rabbit heart (Sigma, Type XI). Partial inhibition of the enzymatic activity of GPI expressed from the mouse neuroleukin cDNA is seen with the rabbit antiserum, but no inhibition of enzymatic activity has been obtained with any of the monoclonal antibodies.

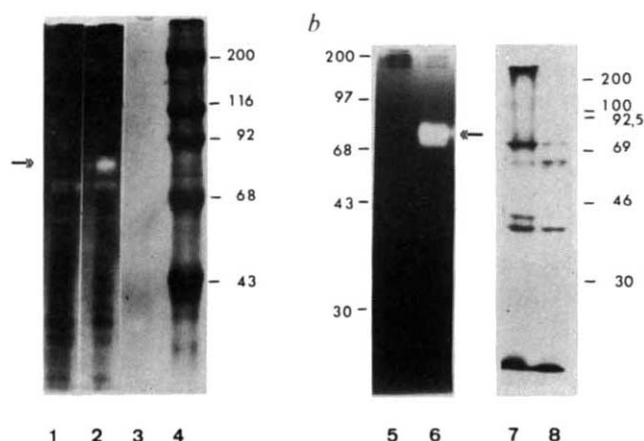
Three hypotheses might explain what we now recognize to be the effect of GPI in our bioassays. First, the enzymatic activity of GPI might account for the biological responses measured, perhaps through modification of either a culture medium component or of a co-released product of COS cells. This hypothesis

is unlikely given that the monoclonal antibodies which block in the bioassay do not affect GPI enzymatic activity. Second, Chaput *et al.* suggest the possibility that GPI may act as a lectin and thus trigger the biological responses measured. Third, the GPI polypeptide or a processed version thereof may be a ligand for a cell surface receptor.

Neuroleukin shares partial sequence homology with a highly conserved domain of the gp120 external envelope glycoprotein of the human immunodeficiency virus type-1 (HIV-1), which led us to identify a toxic effect of gp120 on sensory neurons grown in culture with neuroleukin, but not on neurons responsive to nerve growth factor<sup>5</sup>. The mechanism of gp120 neurotoxicity seen in these studies will need to be re-evaluated in light of the identity of neuroleukin and GPI.

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**Fig. 1** Activation of a membrane protease. Zymograms of schizont membranes were performed after incubation in PBS alone (lane 1) or PBS containing  $1 \mu\text{g ml}^{-1}$  *S. aureus* PI-PLC (lane 2): the new protease activity is arrowed. Lane 3, PI-PLC preparation alone (negative control of proteolytic activity). Lane 4, molecular weight markers (Biorad). **b**, Identification of the new protease. Zymograms (lanes 5, 6) and fluorograms (lanes 7, 8) were performed after immunoprecipitation by monoclonal antibody 31c13 of schizont membrane proteins (lanes 5, 7) or PI-PLC treated schizont membrane proteins (lanes 6, 8). The molecular weight markers are indicated for each gel. In the absence of protease inhibitors and particularly after protease activation following PI-PLC treatment, the proteolytic degradation of p76 to p65 was enhanced<sup>9</sup>.

**Methods.** The FUP strain of *P. falciparum*<sup>22</sup> was cultivated as described<sup>9</sup>. Synchronization was performed by incubation of the cells in 2 volumes of 0.3 M alanine 10 mM HEPES for 3 min at 37 °C (H. Ginsburg personal communication). Schizonts were purified on Percoll-Sorbitol gradients<sup>23</sup>, labelled with <sup>35</sup>S-methionine, and fractionated<sup>9</sup>. The membrane fraction was resuspended in phosphate-buffered saline (PBS) and incubated for 1 h at 37 °C, with 1 to 5  $\mu\text{g ml}^{-1}$  *S. aureus* PI-PLC as indicated above. For immunoprecipitations, the membrane suspension was diluted in 1 volume of 20 mM Tris pH 7.5, 20 mM EDTA, 1 M NaCl, 2% Triton X100; incubated for 30 min at room temperature and centrifuged for 5 min at 12,000 r.p.m. Immunoglobulin 31c13 was purified on Affigel-protein A-MAPS II (Biorad) and thus depleted of the proteases present in the ascitic fluid. Purified IgG was used for immunoprecipitation as described<sup>9</sup> with the following modifications: incubation with antigen was for 1 h at room temperature in the absence of protease inhibitors. For the last wash each sample was divided into two equal volumes. Half of the immunocomplexes were resuspended in Laemmli sample buffer for polyacrylamide gel electrophoresis (SDS-PAGE)<sup>24</sup> and half diluted twofold in solution P (5% SDS, 4% sucrose, 0.2% bromophenol blue) for zymograms. Proteases were analysed electrophoretically in gels containing SDS and 0.1% gelatin<sup>25</sup>. The gelatin-containing gel was processed for assaying protease activity as described<sup>23</sup>.

## Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C

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Membrane anchoring of proteins by a covalently attached glycosyl-phosphatidylinositol moiety has been reported in many different eukaryotic cells including parasite protozoa<sup>1-5</sup>. The diversity of proteins in which this phospholipid attachment is found suggests that it is functionally important and perhaps also functionally pleiotropic. Studies on the Thy-1 antigen of murine lymphocytes<sup>6</sup> indicate that it can facilitate the lateral mobility of membrane proteins. It can also permit the rapid and specific release of the anchored proteins from the membrane following cleavage by a phosphatidylinositol-specific phospholipase C (PI-PLC)<sup>7,8</sup>. Here we show that this type of anchoring may be involved in the regulation of an enzymatic activity. PI-PLC releases a *Plasmodium falciparum* membrane protein of relative molecular mass ( $M_r$ ) 76K (p76) from intact merozoites or isolated schizont membranes and induces a proteolytic activity associated with its soluble form. Endogenous activation of the proteolytic activity of p76 appears to occur at the end of the schizogony and could initiate a cascade of biochemical events associated with merozoite maturation.

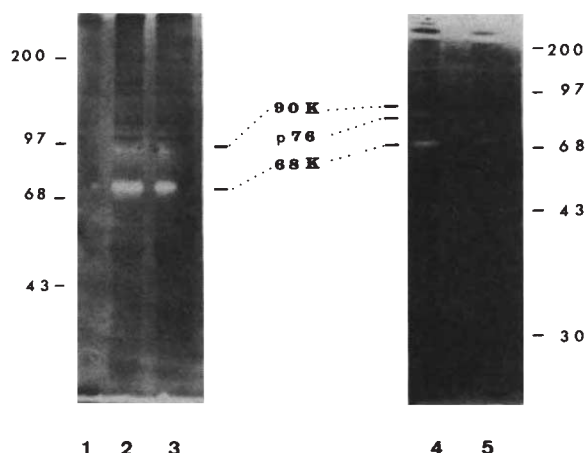
We have previously reported the identification of a series of *Plasmodium falciparum* proteins associated with schizont membranes<sup>9</sup>. Among these, p76 is synthesized in late trophozoites and schizonts as a membrane associated precursor of 83K which is rapidly processed to the 76K polypeptide. The major schizont surface protein (p195) as well as a putative parasite transferrin receptor, were shown to be acylated via 1,2-diacyl-sn-glycerol<sup>4,5</sup> and were therefore candidates for anchorage via a glycosyl-phosphatidylinositol (GPI) moiety. We have recently obtained

direct evidence of this type of anchoring not only for p195 but also for a series of other schizont and merozoite proteins including p76 (C.B.B. *et al.*, in preparation). These proteins can be released from the membrane by PI-PLC cleavage of the GPI linkage and converted to soluble forms.

We detect two main neutral proteolytic activities in schizonts, using gelatin as a substrate (Fig. 1). The lower molecular weight protease was found to be more abundant in the soluble fraction but was also detected in the membrane pellet (Fig. 1, lanes 1 and 2). It migrates as a 75K polypeptide in non denaturing conditions (zymograms) and as a 68K polypeptide in denaturing conditions and probably corresponds to the neutral soluble protease previously described<sup>10-12</sup>. A minor, soluble, 90K protease was also detected in schizonts and merozoites (Fig. 2); this activity was found to be rather unstable. A new neutral

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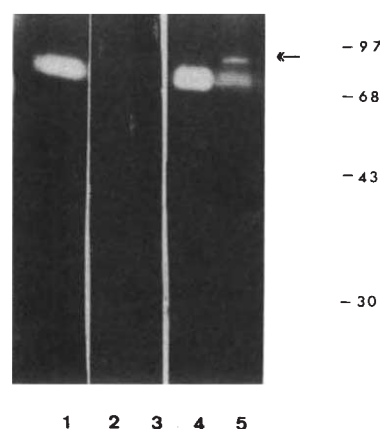


**Fig. 2** Endogenous activation of p76 protease. Electrophoretic analysis of proteases in young schizonts (lane 1), mature schizonts (lane 2), rosaces (lane 3), merozoites (lane 4) and merozoites lysed in the presence of 5 mM pCMPS (lane 5).

**Methods.** Immature schizonts (less than eight nuclei) purified on Percoll-Sorbitol gradients<sup>23</sup> were incubated in culture medium at 37 °C. Their maturation was followed in Giemsa stained smears of the culture. Aliquots were taken at different times corresponding to young schizonts (two to eight nuclei), mature schizonts (more than eight nuclei), or rosaces (schizonts with individualized merozoites). After merozoite release, merozoites were concentrated from the culture supernatant as described<sup>26</sup>. The cells were lysed in 1 volume of cold bi-distilled water and the lysates resuspended in 1 volume of solution P for electrophoretic analysis of proteases (as described in Fig. 1).

proteolytic activity was detected but only when schizont membranes were exposed to *Staphylococcus aureus* PI-PLC (Fig. 1, lane 2). The same results were obtained when *Trypanosoma brucei* PI-PLC was added to a 1% Triton X100 extract of *P. falciparum* schizonts (data not shown). Using a specific monoclonal antibody we have demonstrated that this new protease activity is associated with the previously described p76. Monoclonal antibody 31c13 immunoprecipitates polypeptides p41, p76 and its 83K precursor<sup>9</sup>. As shown in Fig. 1, 31c13 immunoprecipitated an active protease from PI-PLC treated membranes (lanes 6, 8; in non-denaturing conditions, p76 migrates as a 85K polypeptide) and an inactive 76K polypeptide from nontreated membranes (lanes 5, 7). Detergent extraction of membrane proteins did not solubilize an active form of p76 protease although activity could be detected if the membrane preparations were treated with PI-PLC either before or after detergent-solubilization (data not shown).

To investigate whether this novel mechanism of enzyme activation was developmentally regulated we looked for endogenous activation of the p76 protease in *in vitro* cultured synchronous parasites (Fig. 2). Endogenously activated p76 protease was observed only in late schizogony, whereas inactive p76 was already present in mature trophozoites. The proteolytic activity detected in merozoites in the absence of added PI-PLC was, however, lower than that induced by PI-PLC treatment. Furthermore, p76 protease was detected only when the parasites were lysed by osmotic shock and not when they were directly extracted in SDS sample buffer. Endogenous activation was inhibited by p-chloromercuriphenylsulphonate (pCMPS), a PI-PLC inhibitor<sup>13</sup> (Fig. 2, lane 5). These results suggest the existence of a compartmentalized endogenous PI-PLC in mature schizonts and merozoites that does not have access to p76, a situation also described in *Trypanosoma brucei* for the VSG lipase<sup>14</sup>. The role of PI-PLC in the activation of the protease activity of p76 should be investigated at developmental stages of the parasite



**Fig. 3** Activation of p76 protease on intact merozoites. Free merozoites were incubated in PBS (lanes 1, 2) or in PBS + PI-PLC (lanes 4, 5). After incubation, the merozoite pellet and the incubation mixture were separated by centrifugation. Zymograms were performed with the pellet of merozoites incubated in PBS (lane 1), the supernatant of this preparation (lane 2), the pellet of merozoites incubated in PBS + PI-PLC (lane 4), and the supernatant of this preparation (lane 5). Lane 3 corresponds to molecular weight markers (BRL). The p76 protease activity is arrowed.

**Methods.** The *P. falciparum* FUP strain culture was synchronized, schizonts were purified and labelled with <sup>35</sup>S methionine for 4 h at 37 °C as described in Fig. 1. The cells were washed and resuspended in culture medium at 1% haematocrit until merozoite release occurred. Merozoites were prepared<sup>26</sup>, washed, resuspended in 1 volume of PBS and incubated for 30 min at 37 °C in PBS, containing 5 µg ml<sup>-1</sup> *S. aureus* PI-PLC as indicated above. Merozoites were separated from the incubation medium, by a 3 min centrifugation at 10,000 r.p.m. at room temperature. The pellet was resuspended in 1 volume of PBS, and the merozoite suspensions and supernatants were diluted twofold in solution P for electrophoretic analysis of proteases.

life cycle such as maturation and release of merozoites or merozoite-erythrocyte interaction.

In an attempt to determine the localization of p76 in merozoites, we investigated its accessibility to the action of exogenous PI-PLC on intact merozoites (Fig. 3). The p76 protease was specifically solubilized and activated following PI-PLC treatment of intact merozoites. Limited lysis of merozoites occurred during the incubation with PI-PLC, as shown by the presence of some p68 protease activity in the supernatant (lane 5). This could not, however, account for the presence of p76 which was found only in the merozoite supernatant and not in the cell pellet. The observation that exogenous PI-PLC can activate p76 on intact merozoites suggests that the enzyme has access to the GPI anchorage. One possibility is that p76 is on the surface, another is that exogenous PI-PLC can enter the

**Table 1** Inhibition of protease activity by protease inhibitors

|     | Inhibitors    |               |                                     |                                       |                                       |
|-----|---------------|---------------|-------------------------------------|---------------------------------------|---------------------------------------|
|     | PMSF<br>10 mM | EDTA<br>10 mM | Pepstatin<br>50 µg ml <sup>-1</sup> | Leupeptin<br>5/50 µg ml <sup>-1</sup> | Chymostatin<br>50 µg ml <sup>-1</sup> |
| p76 | —             | ++            | ++                                  | +/-                                   | —                                     |
| p68 | ++            | +             | +                                   | ++/+                                  | ++                                    |

Schizont membranes were treated with 5 µg ml<sup>-1</sup> *S. aureus* PI-PLC, resuspended in solution P and analysed for protease activity as described in Fig. 1. Protease inhibitors were added to the 0.1 M glycine buffer in which the gels were incubated after electrophoresis. A control experiment was run in which no inhibitor was added. —, no detectable protease activity. +, faint protease activity. ++, detectable activity but lower than control. ++, protease activity comparable to the control.

the surface, another is that exogenous PI-PLC can enter the rhoptries, organelles which seem to be connected to the merozoite exterior<sup>15</sup>. Indeed recent results of Clark *et al.*<sup>16</sup> and the results obtained for immunofluorescence studies using monoclonal antibody 31c13 (L. Perrin, personal communication) support the latter hypothesis.

Inhibition of the p76 protease by PMSF and chymostatin (Table 1) suggests that it is a serine protease. Leupeptin also inhibited p76 protease whereas pepstatin and EDTA did not. Under these conditions the 68K protease was inhibited by leupeptin and partially by pepstatin and EDTA, displaying properties that are consistent with those previously described<sup>17</sup>. Using the electrophoretic analysis of the proteases in gels containing gelatin, we did not detect any difference in the activities of p76 and p68 over a range of pH from 7.4 to 8.3 (data not shown).

Our demonstration that cleavage of a phosphatidylinositol membrane anchor can lead to enzymatic activation of the released protein identifies a novel function for this type of anchorage. The timing of activation is dependent on accessibility to endogenous PI-PLC and, in the case of *P. falciparum* p76, is related to merozoite development. Given that p76 appears to be a serine protease, it is interesting that a serine protease seems to be involved in the process of red blood cell invasion by malaria merozoites. Invasion can be blocked by serine protease inhibitors<sup>18,19</sup> and the inhibition partially reversed by pretreatment of the red blood cells with chymotrypsin, but not by exposure to a supernatant or an extract of *in vitro* cultured schizonts harvested at the time of merozoite release<sup>19</sup>. Furthermore, studies using *P. knowlesi* merozoites have shown that the serine protease implicated in red blood cell invasion is insensitive to leupeptin<sup>20</sup>. Whether the p76 leupeptin-sensitive serine protease described here is involved in invasion remains to be tested. But the observation that monoclonal antibody 31c13 inhibits the *P. falciparum in vitro* culture<sup>21</sup> supports the hypothesis that this serine protease plays an important role in merozoite maturation.

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## Human gene expression first occurs between the four- and eight-cell stages of preimplantation development

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The earliest stages of development in most animals, including the few mammalian species that have been investigated, are regulated by maternally inherited information<sup>1</sup>. Dependence on expression of the embryonic genome cannot be detected until the mid two-cell stage in the mouse<sup>2-4</sup>, the four-cell stage in the pig (J. Osborn & C. Polge, personal communication), and the eight-cell stage in the sheep<sup>5</sup>. Information about the timing of activation of the embryonic genome in the human is of relevance not only to the therapeutic practice of *in vitro* fertilization and embryo transfer (IVF), but more importantly for the successful development of techniques for the preimplantation diagnosis of certain inherited genetic diseases<sup>6-8</sup>. We describe here changes in the pattern of polypeptides synthesized during the pre-implantation stages of human development, and demonstrate that some of the major qualitative changes which occur between the four- and eight-cell stages are dependent on transcription. In addition, it appears that cleavage is not sensitive to transcriptional inhibition until after the four-cell stage.

The polypeptides synthesized by single human oocytes or cleaving pre-embryos (the term pre-embryo is used in accordance with the recommendations of the MRC/RCOG Voluntary Licensing Authority to describe the conceptus up to 14 days of development<sup>9</sup>) at various times after insemination were labelled by incubation in <sup>35</sup>S-methionine and then separated in one-dimension on 10% polyacrylamide gels. Representative patterns produced are shown in Fig. 1. No consistent differences are detected between the unfertilized egg and the two-cell stage (lanes 2-5), but from the four-cell stage onwards a number of qualitative changes are evident. Five protein bands which are representative of these changes are indicated (A-E). During the four-cell stage (lane 6) there is a relative decrease in the synthesis of polypeptides migrating at a relative molecular mass ( $M_r$ ) of 36K (A) and 50K (D). The eight-cell pre-embryo (lane 7) shows no synthesis of band A and a decreased synthesis of a polypeptide band at 40K (B). New polypeptide complexes appear at 45K (C) and 75K (E). This loss and gain of bands that occurs between the four-cell stage and the morula is consistent in repeated experiments (proteins from six unfertilized eggs; seven pronucleate eggs; six 2-cells, ten 4-cells and ten 8-cell pre-embryos; and six blastocysts were examined) but does not occur in ageing unfertilized oocytes labelled at equivalent times<sup>10</sup> (Fig. 2). A similar transformation in the pattern of protein synthesis takes place during the two-cell stage of mouse embryogenesis<sup>11</sup> and is due to loss of maternal transcripts and the earliest expression of the embryonic genome<sup>3,4</sup>. In the mouse, blockade of transcription by alpha amanitin causes arrest at the two-cell stage<sup>2</sup> and failure to synthesize new proteins but does not arrest the decline in maternal transcript activity<sup>3</sup>. Therefore we examined the effect of alpha amanitin on both cleavage and the polypeptide synthetic profile of human pre-embryos. Figure 3 shows the developmental effects of exposure of human oocytes and pre-embryos to alpha amanitin for varying periods. Two human oocytes incubated in alpha amanitin prior to insemination were fertilized and cleavage proceeded to the four-cell stage but no further in the continuing presence of the transcriptional inhibitor (a). Pronucleate (b, c, d) or two-cell stage pre-embryos

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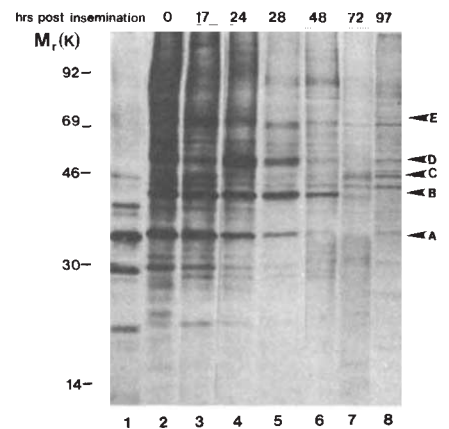


**Fig. 1** Composite fluorograph of  $^{35}\text{S}$ -labelled polypeptides synthesized over a 3h labelling period by single unfertilized oocytes (lanes 2, 4) and pre-embryos at the pronucleate (lane 3), two-cell (lane 5), four-cell (lane 6), eight-cell (lane 7) and blastocyst stages (lane 8) of preimplantation development, and separated by one dimensional polyacrylamide gel electrophoresis. The pattern for early two-cell mouse pre-embryos is shown for comparison (lane 1).

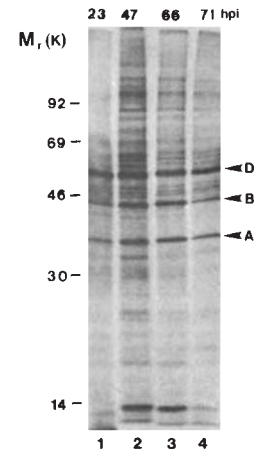
**Methods.** Preimplantation human pre-embryos were donated for research from a therapeutic IVF programme, or were obtained from eggs donated by patients undergoing sterilization<sup>18</sup> or surplus to a GIFT procedure. In the sterilization cohort, induction of ovulation was achieved with clomiphene (Clomid, Merrell) alone followed by 5,000 i.u. hCG administered 34 h before the laparoscopic procedure. For therapeutic IVF/GIFT patients induction of ovulation was achieved with human menopausal gonadotrophin (hMG; Pergonal, Serono) 150 i.u. or 225 i.u. administered daily from day 2 or 3 of the patient's menstrual cycle according to a fixed schedule<sup>19</sup>. hCG (10,000 units) was administered on day 11 of the cycle 34–36 hours before oocyte retrieval. Oocytes were aspirated from the follicles laparoscopically under general anaesthesia, a gas mixture of 5%  $\text{CO}_2$ , 5%  $\text{O}_2$  and 90%  $\text{N}_2$  being used to maintain abdominal distension<sup>20</sup>. On aspiration the oocytes were maintained at 37 °C in Earles Balanced Salt solution (EBS) buffered with HEPES (HEBS)<sup>20</sup> before transfer to the laboratory. Fertilization was achieved using 100,000–150,000 spermatozoa in 1 ml drops of EBS containing 5 mg  $\text{ml}^{-1}$  Fraction V bovine serum albumin (BSA, Sigma) under light paraffin oil in 5%  $\text{CO}_2/95\%$  air. The presence of pronuclei 14–27 h after insemination (hpi) was taken as an indication of fertilization, after which the pre-embryos were transferred to 100  $\mu\text{l}$  drops of EBS containing 10% heat-inactivated human serum (HIS) for further culture. Individual pre-embryos were washed in EBS+BSA, labelled for 3 h in 50  $\mu\text{l}$  drops of EBS+BSA containing 5  $\mu\text{l}$  of high specific activity  $^{35}\text{S}$ -methionine (15  $\text{mCi ml}^{-1}$ , Amersham), washed in EBS and analysed by SDS gel electrophoresis<sup>21,22</sup> on 10% gels. Gels were prepared for fluorography<sup>3,4</sup> and exposed for between 2 and 12 weeks.

(e) exposed continuously to the same concentration of the inhibitor were also able to divide to the four-cell stage. In contrast, four-cell pre-embryos placed in alpha amanitin at different times after insemination (f–l) either remained arrested at this stage (g, k, l), or at most, completed one further complete round of cleavage divisions (f, i, j). Figure 4 shows the gel profiles of polypeptides synthesized by pre-embryos cultured for 30 hours in the presence (lanes 2, 4, 6) or absence (lanes 1, 3, 5) of alpha amanitin. Untreated pre-embryos synthesized polypeptides characteristic of morulae (the 'late' pattern, see lanes 7 and 8, Fig. 1), whereas those pre-embryos treated with alpha amanitin not only failed to generate this late pattern but synthesized only some of the polypeptides seen in the early cleavage stages (the 'early' pattern; see lanes 2–5, Fig. 1).

Human preimplantation embryonic material is difficult to obtain and of variable quality and normality. This paucity of material forces us to draw conclusions from fewer pre-embryos than is ideal. Nevertheless our data are consistent and taken together suggest that the first two cell cycles of human embryogenesis are regulated at a post-transcriptional level, utilizing maternally inherited information, and that activation of the embryonic genome takes place between the four- and eight-cell stages and is essential if both synthesis of proteins and further cleavage are to occur. This conclusion is consistent with the findings of Tesarik *et al.*<sup>12,13</sup> who could not detect autoradiographically the extranucleolar incorporation of radioactive uridine before the four-cell stage, or nucleolar labelling before the third cleavage division (six- to eight-cell stage). The interpretation of our finding that development beyond, but not up to, the four-cell stage is sensitive to alpha amanitin is complicated by the observation that some *untreated* pre-embryos spontaneously arrested at the four-cell stage (Fig. 3b, c) or the eight-cell stage (Fig. 3g). This propensity for spontaneous arrest of cleavage at around this time in the human has been reported by us previously<sup>14</sup> although it may not occur in embryos derived from unstimulated cycles (J. C. Osborn, personal communication). It is likely to be aggravated in these experiments by the fact that the pre-embryos used were, in the majority of cases, 'spares' from a therapeutic IVF programme and therefore by definition the remainder from a cohort of pre-embryos from which the 'best' three had been selected for transfer. Although this makes individual experiments difficult to control, over a number of experiments there was a clear tendency for untreated pre-embryos to progress further than the pre-embryos exposed to the transcriptional inhibitor. A high incidence of spontaneous cleavage arrest also occurs around the time of gene activation in the mouse<sup>15</sup> the pig<sup>16</sup>, and the sheep<sup>5</sup> and may signify an



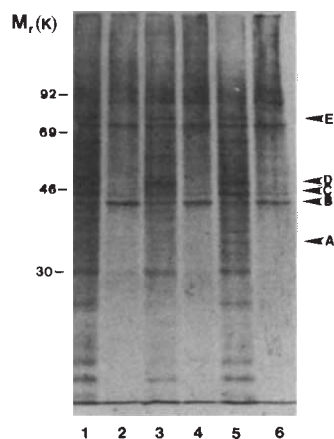
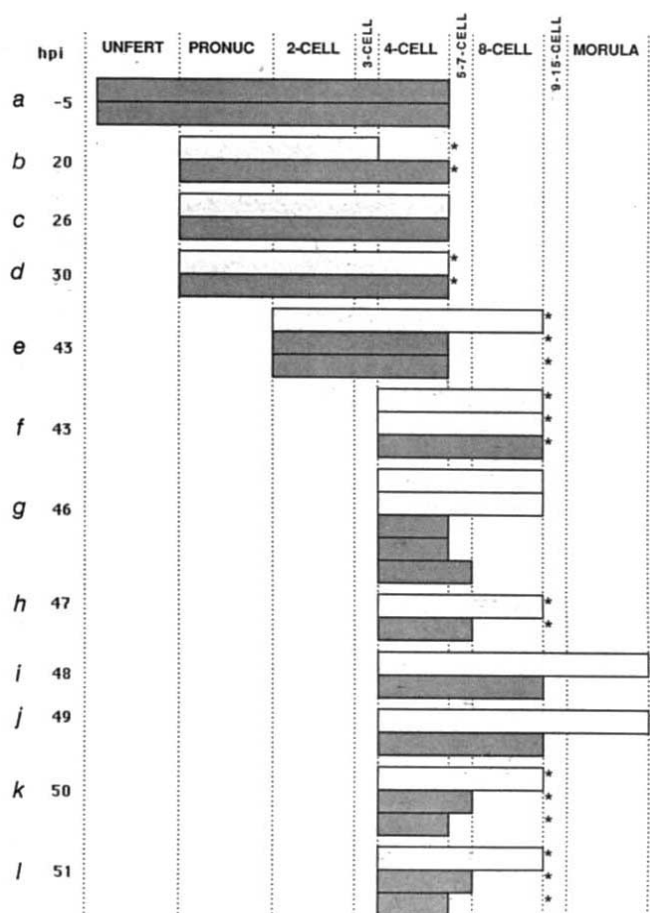
**Fig. 2** Fluorograph of  $^{35}\text{S}$ -labelled polypeptides synthesized by individual human oocytes which failed to fertilize *in vitro*. Oocytes were inseminated *in vitro* with 100,000–150,000 sperm  $\text{ml}^{-1}$ , 4–5 h after aspiration from the follicles as part of a therapeutic IVF programme. Oocytes which failed to show the presence of two pronuclei within 24 h of insemination were designated as unfertilized and labelled for 2 h in EBS+BSA containing  $^{35}\text{S}$ -methionine at 23 h (lane 1), 47 h (lane 2), 66 h (lane 3) and 71 h (lane 4) after insemination. Bands A, B, and D correspond with those in Fig. 1.



increased sensitivity to insult *in vitro* during this period. The fact that some alpha amanitin-treated four-cell pre-embryos cleaved once to the eight-cell stage but no further suggests that gene activation may be initiated in the early to mid four-cell stage. This situation is again analogous to that in the sheep<sup>5</sup> where a few eight-cell stages can proceed to the 16-cell stage in the presence of alpha amanitin, and to that in the mouse where exposure to alpha amanitin within 8–9 hours after the first cleavage division blocks further development, whereas exposure to the drug after this time allows a single cleavage but no more<sup>4,17</sup>. Also, as in the mouse, alpha amanitin blocked the appearance of 'late' new proteins, but did not block the loss of 'early' proteins, suggesting that in the mouse and the human, loss of maternal template messenger RNA occurs at around the same time as, but independently of, gene activation<sup>3</sup>.

Our findings have important consequences for the development of certain techniques for the preimplantation diagnosis of genetic disorders. Diagnosis may be attempted either by using specific DNA probes to identify directly missing or defective gene sequences, or by measuring the products of the defective gene<sup>6</sup>. If the latter technique is to be used for preimplantation diagnosis, then it is essential that the product identified is encoded by the embryonic genome rather than being maternally inherited in the oocyte. The knowledge that gene activity first occurs between the four- and eight-cell stages of human development means that attempts to use this form of diagnosis should be made on blastomeres or tissue from pre-embryos after this developmental stage.

**Fig. 3** Results of 12 experiments (a-l) in which individual human pre-embryos were cultured *in vitro* in the presence (dark bars) or absence (light bars) of alpha amanitin. Oocytes were fertilized *in vitro* and then transferred to 100  $\mu$ l drops of EBS+HIS containing alpha amanitin (100  $\mu$ g ml<sup>-1</sup>) at various times after insemination (hpi). Development of each pre-embryo was assessed approximately every 12 h. In those experiments marked \* further development of the pre-embryos was stopped by the experimenter for labelling in <sup>35</sup>S-methionine and gel electrophoresis or for HPRT enzyme analysis<sup>6</sup>. In experiment a freshly aspirated oocytes were incubated in alpha amanitin (100  $\mu$ g ml<sup>-1</sup>) for 5 h before insemination.



**Fig. 4** Fluorograph of radiolabelled polypeptides synthesized by human pre-embryos (43 hpi) labelled with <sup>35</sup>S-methionine after exposure to alpha amanitin for 30 h. Two late two-cell pre-embryos and one early four-cell pre-embryo (43 hpi) surplus to a transfer procedure were placed in 100  $\mu$ l drops of EBS+HIS containing alpha amanitin (100  $\mu$ g ml<sup>-1</sup>) for 30 h (lanes 2, 4, 6). An equivalent number of pre-embryos from the same patient (one at the late two-cell stage and two at the early four-cell stage) were cultured in the absence of the inhibitor (lanes 1, 3, 5). At the end of the experimental period the morphological stage of development of each pre-embryo was assessed (see Fig. 3, lanes 5, 6) before they were labelled for 3 h in EBS+BSA containing <sup>35</sup>S-methionine and processed for electrophoresis as for Fig. 1.

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# The presence of malformed proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins

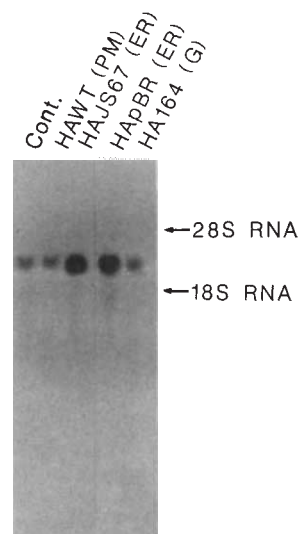
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Two glucose-regulated proteins, GRP78 and GRP94, are major constituents of the endoplasmic reticulum (ER) of mammalian cells. These proteins are synthesized constitutively in detectable amounts under normal growth conditions; they can also be induced under a variety of conditions of stress including glucose starvation and treatment with drugs that inhibit cellular glycosylation, with calcium ionophores or with amino-acid analogues<sup>1,2</sup>. Unlike the closely-related heat shock protein (HSP) family<sup>2-4</sup>, the GRPs are not induced significantly by high temperature. Recently, GRP78 has been identified as the immunoglobulin heavy chain binding protein (BiP) (ref. 5 and Y.K. *et al.*, in preparation) which binds transiently to a variety of nascent, wild-type secretory and trans-membrane proteins and permanently to malformed proteins that accumulate within the ER<sup>6-8</sup>. We have tested the hypothesis that the presence of malformed proteins may be the primary signal for induction of GRPs by expressing wild-type and mutant forms of influenza virus haemagglutinin (HA) in simian cells. Only malformed HAs, whose transport from the ER is blocked, induced the synthesis of GRPs 78 and 94. Additional evidence is presented that malforming *per se*, rather than abnormal glycosylation<sup>1</sup>, is the proximal inducer of this family of stress proteins.

Because the majority of conditions that cause induction of GRPs interfere directly or indirectly with glycosylation<sup>1,9</sup>, it has been suggested<sup>9</sup> that the common stimulus for the induction may be the presence of underglycosylated proteins in the ER. However, the fact that GRPs are also induced by treatment with amino-acid analogues<sup>10-12</sup> is difficult to reconcile with this theory. An alternative hypothesis, strengthened by the observed interaction between GRP78 and mutant polypeptides<sup>8</sup>, is that induction of GRPs is triggered by the presence of malformed proteins within the ER. To test this idea, we have examined the induction of GRP78 in cells which express malformed forms of HA that accumulate within the ER but do not differ at all in their pattern of glycosylation from the nascent wild-type protein. These mutants include HAJ67, in which substitution of residue Cys67 by Ser prevents the formation of one of the six disulphide bonds in the HA molecule (M.S. *et al.*, in preparation), and HApBR<sup>13</sup> which contains a six-residue extension of the cytoplasmic tail of the molecule. For comparison, we have examined the induction of GRP78 in cells expressing the wild-type HA<sup>14</sup> or HA164<sup>13</sup>, another cytoplasmic tail mutant that accumulates in a post-Golgi compartment rather than in the ER.

CV-1 cells were infected with high-titre recombinant SV40 viruses encoding the wild-type or mutant HA proteins. Forty-eight hours after infection, the level of GRP78 messenger RNA in each infected culture was determined by isolating total mRNA samples and analysing them by Northern hybridization using <sup>32</sup>P-labelled GRP78 complementary DNA as a probe (Fig. 1). Infected cultures were also labelled with <sup>35</sup>S-methionine at various times after infection and the levels of synthesis of the GRP78 polypeptide, of the wild-type or mutant HA glycoprotein and of the SV40 capsid protein VP1 were examined by autoradiography following separation of the labelled proteins by SDS-PAGE (Fig. 2a). Immunoprecipitation with the



**Fig. 1** Effect of the expression of wild-type and mutant HAs on the level of GRP78 mRNA in CV-1 cells. CV-1 cells were mock infected or infected<sup>13</sup> with SV40-HA recombinant viruses SVEHA3<sup>14</sup>, SVHApBR<sup>13</sup>, SVHA164<sup>13</sup> and SVHAJS67 (M.S. *et al.*, in preparation). Total cellular RNAs were isolated<sup>26</sup> 48 h after infection. Samples corresponding to equal numbers of cells were electrophoresed on a 1% formaldehyde-agarose gel<sup>26</sup>, blotted onto a nitrocellulose membrane and hybridized with nick-translated GRP78 cDNA (Y.K. *et al.*, in preparation) at 68 °C in 6×SSC, 5× Denhardt's solution and 0.1% SDS. The membrane was washed with 2×SSC and 0.1% SDS at 68 °C. Under the hybridization conditions used in this experiment, the GRP78 cDNA does not bind to HSP70 sequences (data not shown).

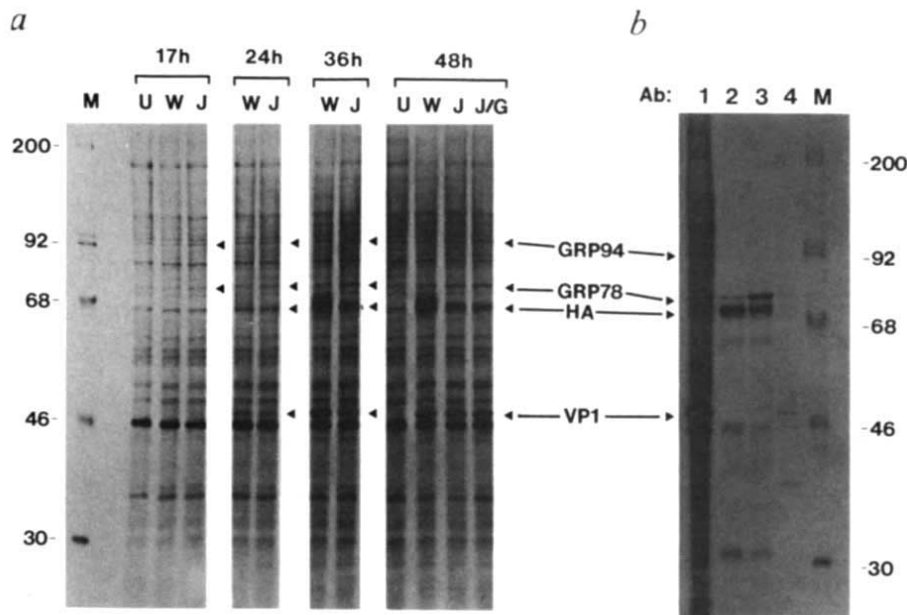
appropriate specific antiserum confirmed the identity of these proteins (Fig. 2b and results not shown). The association between GRP78 and the mutant HA polypeptide is indicated by the co-precipitation of the two proteins by specific antisera directed against each individual species<sup>8,27</sup> (Fig. 2b).

Significantly increased amounts of GRP78 mRNA were detected in cells expressing the ER-blocked mutants, HAJ67 or HApBR, compared to the low, constitutive level present in mock-infected cells (Fig. 1). The steady-state level of GRP78 mRNA was, however, no greater in cells expressing large amounts of wild-type HA (see Fig. 2) than in mock-infected cells. Similarly, mutant HA164, which accumulates at a post-Golgi stage of the secretory pathway, had no effect on the synthesis of GRP78 mRNA (Fig. 1). The time course and efficiency of infection of the CV-1 cells by the various SV40 viruses can be deduced from Fig. 2a by examining the production of HA polypeptides (relative molecular mass,  $M_r \approx 63$ K) from the recombinant SV40 genome as well as that of the VP1 capsid protein ( $M_r = 48$ K) from the helper virus genome. Synthesis of these late viral proteins in cells infected with vectors encoding wild-type HA or the HAJ67 mutant began ~24 h after infection and reached peak levels at 36 to 48 h when the RNA samples were collected. The time course and level of production of wild-type HA and mutant HAJ67 were very similar. Thus the induction of GRP78 mRNA in cells expressing HAJ67 but not in cells expressing the wild-type HA cannot be explained by variability in the extent of infection of cells by the two recombinant virus stocks.

Induction of GRP78 mRNA is accompanied by increased synthesis of the GRP78 protein (Figs 2, 3). Figure 2a shows an autoradiograph of proteins labelled in a one-hour pulse at various times after monolayers of CV-1 cells were mock infected (U) or infected with recombinant SV40 vectors encoding wild-type HA (W) or mutant HAJ67 (J). An identical experiment was performed with cells infected with vectors encoding mutants

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**Fig. 2** Effect of the expression of wild-type and mutant HAs on the induction of GRPs in CV-1 cells. CV-1 cells were mock-infected or infected<sup>13</sup> with SVEHA3<sup>14</sup> or SVHAJS67 (M.S. *et al.*, in preparation). At various times after infection the cells were labelled with <sup>35</sup>S-methionine for 1 h. *a*, Autoradiograph of the labelled proteins from total cell extracts<sup>13</sup> following separation by electrophoresis on an 8% SDS-polyacrylamide gel. U, uninfected cells; W, cells infected with SVEHA3; J, cells infected with SVHAJS67; J+G, cells infected with SVHAJS67 with glucose maintained at a high concentration by replacement at 12-h intervals with fresh medium containing 25 mM glucose. *b*, Identification of the HA and GRP78 species by immunoprecipitation. CV-1 cells were infected and labelled with <sup>35</sup>S-methionine 48 h post-infection. Total cell extracts were prepared and immunoprecipitated with the following sera: lane 1, no serum; lane 2, a rabbit anti-HA serum prepared against purified HA from the A/Japan/305/57 strain of influenza virus<sup>9</sup>; lane 3, a rat anti-mouse BiP monoclonal antibody<sup>27</sup> (provided by D. Bole, L. Hendershot & J. Kearney, University of Birmingham, Alabama); lane 4, a rabbit anti-mouse GRP78 serum prepared against a fusion protein expressed in *Escherichia coli* from a full-length cDNA encoding murine GRP78. Although this antibody has a high affinity for murine GRP78, it binds only weakly to simian GRP78 from CV-1 cells (Y.K. *et al.*, in preparation). The immunoprecipitated proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and autoradiographed.



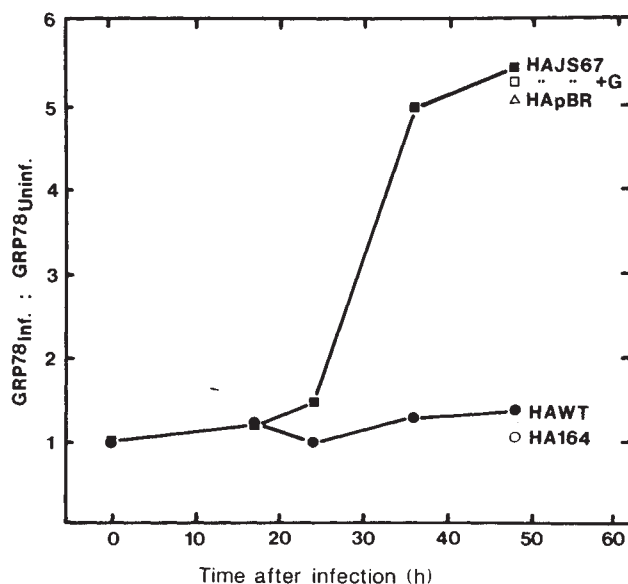
HApBR and HA164 (data not shown). Densitometry was then used to quantitate the relative amounts of labelled GRP78 present in the cell extracts at each time after infection (Fig. 3). The amount of GRP78 protein synthesized in cells expressing the ER-blocked mutants HAJ67 and HApBR was increased significantly over the constitutive levels present in cells expressing wild-type HA, HA164 or in mock-infected cells. The induction of GRP78 began as soon as synthesis of the HAJ67 or HApBR polypeptides could be detected (at 24 h post-infection), and reached a maximum of five- to six-fold at 36–48 h after infection when synthesis of the mutant HA proteins was at its peak. GRP94 was induced to a lesser extent (two-fold) in cells expressing the ER-blocked mutants.

It has been reported<sup>15</sup> that the overproduction of viral glycoproteins can cause depletion of glucose from culture medium and consequent induction of GRPs. We found, however, that the wild-type HA, HA164 and the ER-blocked mutants were produced in approximately equal amounts and that the induction of GRP78 was not abolished by glucose supplementation<sup>15</sup> during infection with SVHAJS67 (Figs 2a and 3). We therefore consider that the observed induction is a direct result of the accumulation of the malformed protein in the ER.

In a second series of experiments, we have studied the effects of several inhibitors of the addition and processing of *N*-linked glycans on the induction of GRPs in uninfected CV-1 cells (Fig. 4). Drugs that interfere with oligosaccharide processing in the Golgi apparatus did not induce the synthesis of GRPs. Thus, neither swainsonine (SW), which inhibits Golgi mannosidase II<sup>16</sup>, resulting in glycoproteins with hybrid oligosaccharides, nor 1-deoxymannojirimycin (dMN), which blocks the conversion of mannose-rich oligosaccharides to complex oligosaccharides by inhibiting Golgi mannosidase I and II<sup>16</sup>, caused induction of the GRP proteins. In contrast, three inhibitors that interfere with glycosylation in the ER caused significant induction of the GRP proteins. Thus treatment with castanospermine (CA), which results in the formation of glycoproteins containing oligosaccharides of the structure Glc<sub>3</sub>Man<sub>7-9</sub>(GlcNAc)<sub>2</sub> by inhibition of the ER-resident glucosidases I and II<sup>16</sup>, caused induction of both GRP78 and the normal, glycosylated form of GRP94. Treatment with 1-deoxynojirimycin (dN), which also inhibits glucosidase I and II<sup>16</sup> and in addition partially blocks *N*-linked glycosylation by inhibiting the formation of dolichyl-PP-

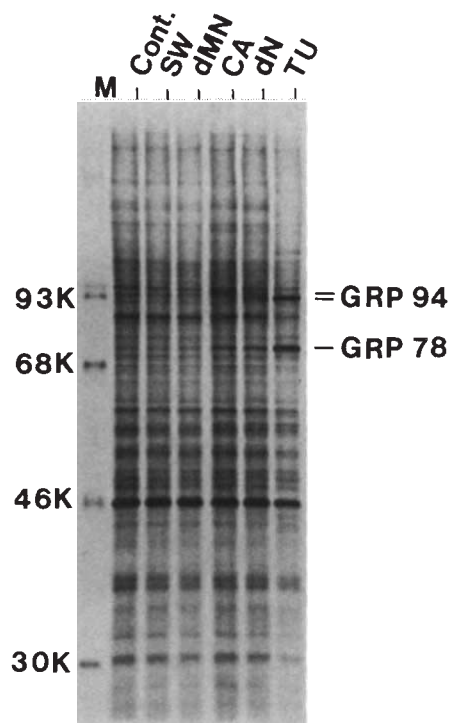
GlcNAc<sup>16,17</sup>, caused increased synthesis of GRP78 and of both the glycosylated and non-glycosylated forms of GRP94. Finally, as reported previously<sup>1,18</sup>, tunicamycin (TU) caused marked induction of GRP78 and the non-glycosylated GRP94. This drug completely blocks *N*-linked glycosylation by inhibition of the formation of dolichyl-PP-GlcNAc<sup>16</sup>.

The three agents (TU, dN and CA) that caused induction of GRPs have all been shown to inhibit or retard the intracellular



**Fig. 3** Time course of induction of GRP78 in cells expressing ER-blocked HA mutants. CV-1 cells were mock infected or infected<sup>13</sup> with SV40-HA recombinant viruses SVEHA3<sup>14</sup>, SVHApBR<sup>13</sup>, SVHA164<sup>13</sup> and SVHAJS67 (M.S. *et al.*, in preparation). At various times after infection the cells were labelled with <sup>35</sup>S-methionine for 1 h before cell extracts were prepared and analysed by SDS-PAGE<sup>13</sup>. Densitometry was used to quantitate the intensity of the GRP78 bands on the autoradiogram (for example see Fig. 2a). The relative ratio of the intensity of the GRP78 band at each time point to that measured in extracts from mock-infected cells was plotted for cells expressing wild-type HA (●), HA164 (○), HApBR (△) and HAJ67 (■).





**Fig. 4** Effect of inhibitors of addition or processing of *N*-linked oligosaccharides on the expression of GRPs. Nearly-confluent CV-1 cells were treated with 6  $\mu$ M tunicamycin (TU), 0.5 mM castanospermine (CA), 5 mM *l*-deoxynojirimycin (dN), 1 mM *l*-deoxymannojirimycin (dMN) or 0.1 mM swainsonine (SW) for 16 h and labelled with  $^{35}$ S-methionine for 1 h in the presence of the same concentration of the inhibitor. Cell extracts were prepared and analysed by SDS-PAGE<sup>13</sup>. The inhibitor concentrations used in this experiment had previously been shown to cause the expected inhibition of oligosaccharide addition or processing of HA expressed in CV-1 cells (ref. 14 and unpublished results).

transport of nascent glycoproteins (reviewed in ref. 16), while the non-inducers (dMN and SW) have no apparent effect on exocytosis<sup>16</sup>. This suggests a correlation between GRP induction and the retention of proteins in the ER. In contrast, we found no correlation between underglycosylation, which was caused by TU and dN but not by CA, and GRP induction, which was caused by all three agents. We suggest that abnormal glycosylation resulting from glucose starvation<sup>1,15</sup>, from treatment with drugs<sup>1,16</sup> or ionophores<sup>1,9</sup>, or from cellular mutations<sup>1,9</sup> indirectly causes induction of GRPs by preventing or delaying the folding and assembly of nascent glycoproteins leading to accumulation of malformed polypeptides in the ER. Other inducing agents, for example amino-acid analogues<sup>10-12</sup>, sulphhydryl-reducing agents<sup>19</sup>, low extracellular pH<sup>19</sup> or prolonged anaerobiosis<sup>20</sup>, could act directly via protein denaturation or cause the synthesis of incorrectly folded or unstable polypeptides.

Our previous observations that BiP/GRP78 binds to prefolded or malformed proteins that accumulate in the ER<sup>8</sup> led in part to the hypothesis that this resident ER protein is involved in the folding and assembly of exocytotic proteins both during normal synthesis and under conditions that promote protein denaturation and aggregation<sup>3,6,8</sup>. Our current results demonstrate that GRPs, like HSPs<sup>3,21,22</sup>, are induced by the presence of malformed protein in the appropriate cellular compartment. We do not know whether the association of GRP78 with unfolded or abnormal polypeptides simply reflects the protein's function in the ER or whether the formation of these complexes in some way initiates the induction process. In either case, a mechanism must exist to monitor events in the lumen of the ER and to transduce signals across the lipid bilayer of the ER to the nucleus. The

nature of the induction signal and the manner in which it is conveyed to the nucleus to cause increased transcription of the GRP genes remain matters for speculation. It is tempting to propose that reduction of the free concentration of GRP78 by its sequestration into protein complexes may provide the primary signal for induction of GRP synthesis, perhaps via the release of calcium bound to the conserved, highly acidic sequences found in all these proteins<sup>5,23,24</sup>. A recent report that GRP94 may cross the ER membrane with its largest domain exposed on the cytoplasmic face<sup>24,25</sup> also raises the possibility that it could interact with GRP78, either before or after the association of GRP78 with unfolded protein, thus promoting the transduction of the stress response to the nucleus.

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## Structure refined to 2 Å of a nicked DNA octanucleotide complex with DNase I

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The cutting rates of bovine pancreatic deoxyribonuclease I (DNase I) vary along a given DNA sequence, indicating that the enzyme recognizes sequence-dependent structural variations of the DNA double-helix<sup>1,2</sup>. In an attempt to define the helical parameters determining this sequence-dependence, we have co-crystallized a complex of DNase I with a self-complementary octanucleotide and refined the crystal structure at 2 Å resolution. This structure confirms the basic features of an early model<sup>3,4</sup>, namely that an exposed loop of DNase I binds in the minor groove of B-type DNA and that interactions do occur with the backbone of both strands. Nicked octamer duplexes that have lost a dinucleotide from the 3'-end of one strand are hydrogen-bonded across a two-fold axis in the crystal to form a quasi-continuous double helix of 14 base pairs. The DNA 14-mer has a B-type conformation and shows substantial distortion of both local and overall helix

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parameters, induced mainly by the tight interaction of Y73 and R38 in the unusually wide minor groove. Directly coupled to the widening of the groove by  $\sim 3\text{\AA}$  is a  $21.5^\circ$  bend of the DNA away from the bound enzyme towards the major groove, suggesting that both DNA stiffness and groove width are important in determining the sequence-dependence of the enzyme cutting rate. A second cut of the DNA which is induced by diffusion of  $\text{Mn}^{2+}$  into the co-crystals suggests that there are two active sites in DNase I separated by more than  $15\text{\AA}$ .

Diffraction intensities were collected to  $2\text{\AA}$  resolution from orthorhombic crystals of space group C222<sub>1</sub> and grown at  $4^\circ\text{C}$  in the presence of EDTA, using polyethylenglycol 6000 as precipitating agent (Table 1). The structure was solved by molecular replacement<sup>5</sup>, using the known coordinates of DNase I without DNA present, and the starting model was refined according to the Hendrickson-Konnert reciprocal least-squares procedure<sup>6</sup> to yield an *R*-factor of 0.187 for a model with very good stereochemistry (Table 1). Details of the crystallization, data collection, structure determination and refinement will be published elsewhere.

High pressure liquid chromatography indicated that the co-crystals contain a 1:1 mixture of the original self-complementary octanucleotide d(GCGATCGC) and a hexamer, suggesting that a nicked double-strand is bound to DNase I, despite the presence of 15mM EDTA in the crystallization medium. The difference electron density map, however, only showed seven base pairs instead of the expected eight, and furthermore revealed that one phosphate was missing. The only consistent explanation for these observations, confirmed during refinement, is that DNase I removes a dinucleotide (pGpC) from the 3' end of one of the strands of the octamer duplex before crystallization. In the crystal, the nicked double-strands dimerize across a twofold axis with base pair formation between the newly formed single-stranded regions producing a quasi-continuous double helix of 14 base pairs (bp). The packing diagram shown in Fig. 1 illustrates that it is exactly a 14-mer, that can be accommodated in the C222<sub>1</sub> crystals because the 5'-terminal Gs stack on to H118 residues of twofold related DNase I molecules.

Apart from a few side chains in direct contact with DNA, no conformational change occurs in DNase I on binding of the oligonucleotide, and the  $C_\alpha$ -positions of the free and the DNA-bound enzyme can be superimposed with an r.m.s. difference of  $0.37\text{\AA}$ . Besides H118, the only significant shifts in  $C_\alpha$ -positions (about  $1\text{\AA}$ ) are found in the exposed loop around Y73 which interacts tightly in the DNA minor groove and in the helix-turn region V43 to P57. These regions are stabilized through the contacts with DNA, as is obvious from their low isotropic temperature factors. On the other hand, the carbohydrate side-chain and the region around the short disulphide bridge (C98-C101) show even higher disorder than in the parent structure, confirming the biochemical evidence that neither is important for binding and cutting of DNA (ref. 7).

In contrast to the remarkably constant protein, the DNA appears to be significantly distorted, highlighting the intrinsic flexibility of DNA (refs 8-11) and hence the difficulty in predicting its interaction with protein. Although the local helix parameters<sup>12,13</sup> calculated by comparing the orientation of neighbouring base pairs vary substantially in the DNase I-octanucleotide complex, they clearly show that the oligonucleotide has the B-type conformation and gives the low mean slide value ( $0.2\text{\AA}$ ) characteristic of a B-type stacking pattern of bases, together with a typical mean rise per base pair ( $3.5\text{\AA}$ ), average intrastrand phosphate distance ( $6.6\text{\AA}$ ), large variation of sugar conformation, and orientation of phosphate groups relative to the grooves. Some individual helix parameters are affected by protein contacts, the main distortion being induced by the unusual stacking interaction of Y73 with the deoxyribose ring of T313 (see Fig. 2, also for nomenclature). It flips the torsional angles around the C4'-C5' and C5'-O5' bonds from the usual *gauche/trans* to a *trans/gauche* conformation, and at the same

Table 1 Data collection and refinement

|                                                                                                              |                  |                           |                            |                             |                      |
|--------------------------------------------------------------------------------------------------------------|------------------|---------------------------|----------------------------|-----------------------------|----------------------|
| Crystals space group C222 <sub>1</sub> , $a = 72.9\text{\AA}$ , $b = 100.1\text{\AA}$ , $c = 92.6\text{\AA}$ |                  |                           |                            |                             |                      |
| Statistics of data collection                                                                                |                  |                           |                            |                             |                      |
|                                                                                                              | Resolution       |                           |                            |                             |                      |
|                                                                                                              | ( $\text{\AA}$ ) | $N$ ( $F > 3\sigma(F)$ )* | $N_{\text{theor}}^\dagger$ | $R_{\text{merge}}^\ddagger$ |                      |
| Diffraction meter                                                                                            | 10-3.0           | 6,870(5,207)              | 6,870                      | 0.108                       |                      |
| Oscillation film                                                                                             | 10-2.0           | 21,350 (18,019)           | 22,301                     | 0.086                       |                      |
| Final refinement parameters                                                                                  |                  |                           |                            |                             |                      |
| No. of cycles                                                                                                |                  |                           |                            |                             | 80                   |
| No. of variables                                                                                             |                  |                           |                            |                             | 7,703                |
| No. of atoms (non-hydrogen)                                                                                  |                  |                           |                            |                             | 2,545                |
| No. of water molecules                                                                                       |                  |                           |                            |                             | 212                  |
| r.m.s. deviation of bond length ( $\text{\AA}$ )                                                             |                  |                           |                            |                             | 0.018                |
| r.m.s. deviation of bond angles (degrees)                                                                    |                  |                           |                            |                             | 3.1                  |
| Resolution                                                                                                   |                  |                           |                            |                             | 6.0-2.0 $\text{\AA}$ |
| No. of $F_{\text{obs}}$ ( $> 3\sigma(F_{\text{obs}})$ )§                                                     |                  |                           |                            |                             | 17,373               |
| <i>R</i> -factor (initial) $3\sigma$ data                                                                    |                  |                           |                            |                             | 0.341                |
| <i>R</i> -factor (final) $3\sigma$ data                                                                      |                  |                           |                            |                             | 0.187                |
| <i>R</i> -factor (final) all data                                                                            |                  |                           |                            |                             | 0.207                |

Diffraction data were collected from two crystals on an Enraf Nonius CAD4 diffractometer and corrected for geometrical factors, radiation damage and absorption, using the MRC-program package. The oscillation film dataset was obtained from 7 crystals and consists of 61 film packs (3 films per pack), which were processed using MOSFLM (Imperial College London) and the Daresbury CCP4 program system. The starting model for refinement was obtained by molecular replacement using the known coordinates of DNase I (ref. 3) and observed  $3\text{\AA}$  amplitudes of the co-crystals. The orientation and position of DNase I in the orthorhombic cell were determined with the fast rotation function<sup>29</sup> and an *R*-factor search respectively, yielding unequivocal solutions. 12 cycles of CORELS rigid body refinement<sup>30</sup> decreased the *R*-factor from 0.42 to 0.33 for  $3\sigma$  reflections between 10 and  $3\text{\AA}$ . A piece of DNA was built into a  $F_0 - F_c$  difference density map, and the resulting model was refined using an FFT version of the PROLSQ program<sup>6</sup>.

\* $N$ , total number of reflections in resolution shell

$^\dagger N_{\text{theor}}$ , maximum number of reflections possible

$^\ddagger R_{\text{merge}} = \sum \sum |I_{ij} - \bar{I}_i| / \sum_j \bar{I}_i$

§, Oscillation film data

time affects the base orientation, resulting in a high individual roll angle of the T313/C314 step ( $13.1^\circ$ ). The unusually low helix-twist angle ( $20.8^\circ$ ) between base pairs C302-G301' and G303-C314, on the other hand, appears to be mainly a consequence of the missing phosphate. Overall the conformation of the DNA also deviates from ideal geometry in showing a  $21.5^\circ$  bend towards the major groove and away from the bound enzyme. The largest contribution to this bend comes from step A304-T313/G303-C314 with an angle of  $9.6^\circ$  between average base pair plane normals. This bending and the simultaneous widening of the minor groove to  $\sim 15\text{\AA}$  (compared to  $12\text{\AA}$  in canonical B-DNA) result from the tight interaction of residues Y73 and R38, which completely fill the minor groove (Fig. 2).

Besides the three side chains interacting in the minor groove, either through stacking (Y73) or hydrogen bonding to oxygen atoms of pyrimidines (R38, R9), there are ten residues forming hydrogen bonds and another three residues (T10, S72, P134) forming van der Waal's contacts to the backbone of both DNA strands (Fig. 2). Surprisingly, only one phosphate group forms a salt-bridge (with R108), whereas five others are hydrogen-bonded to either backbone NH groups (S40, E13 and S14) or side-chain  $\text{NH}_2$ - or OH groups. In an uncleaved DNA substrate the scissile phosphodiester bond would presumably form an additional ion pair with R9 as this residue is located directly at the nick in our complex. Direct contacts with the protein exist on one side of the DNA only, extending over a stretch of 6 bp plus two adjacent phosphates. There are no contacts in the major groove to the 5' side of the cleaved bond, but the protein is approaching the DNA backbone again some 5 bp away in the region around T174, thereby restricting access to the major



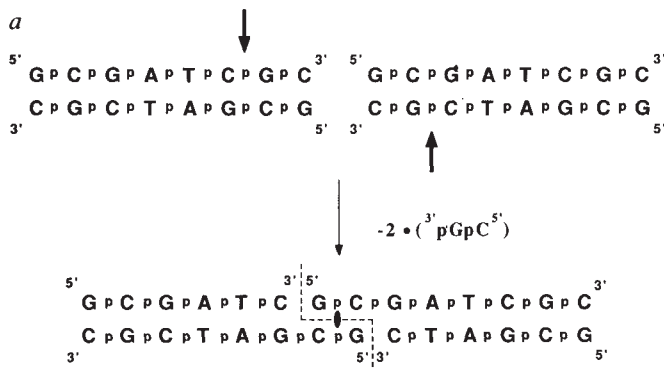


Fig. 1

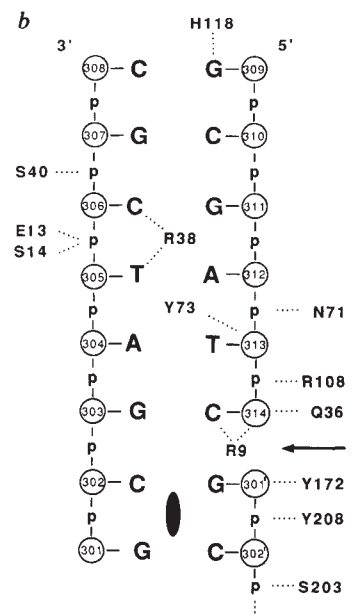
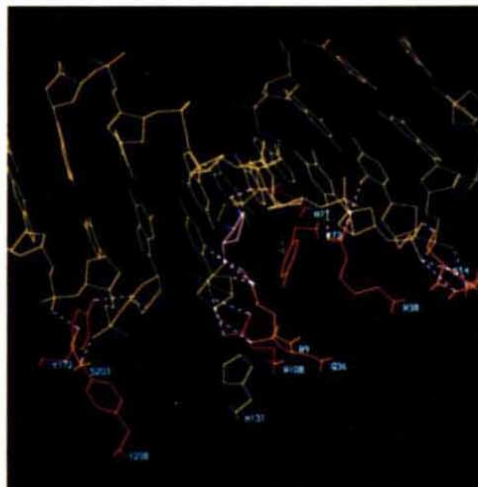
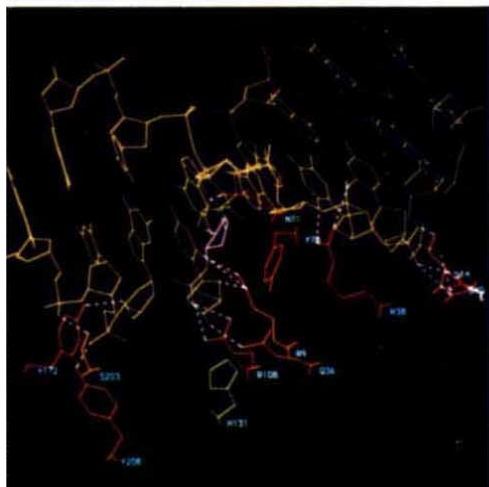
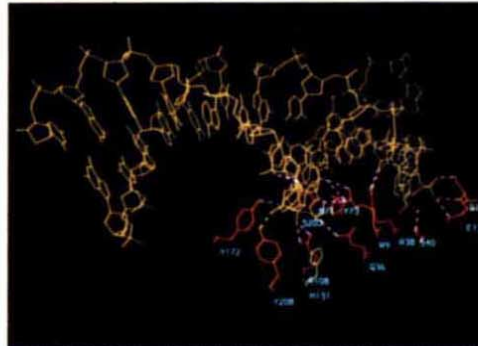
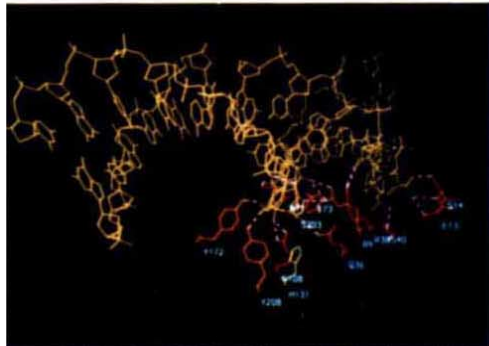
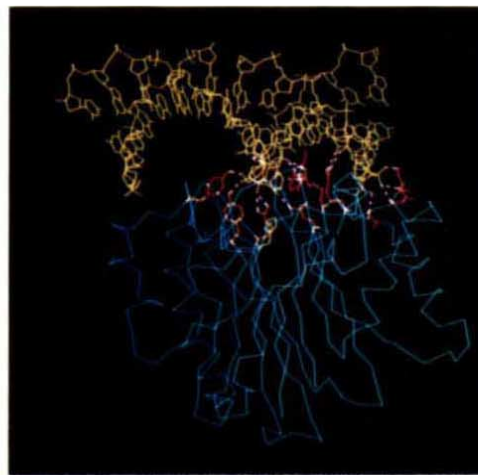
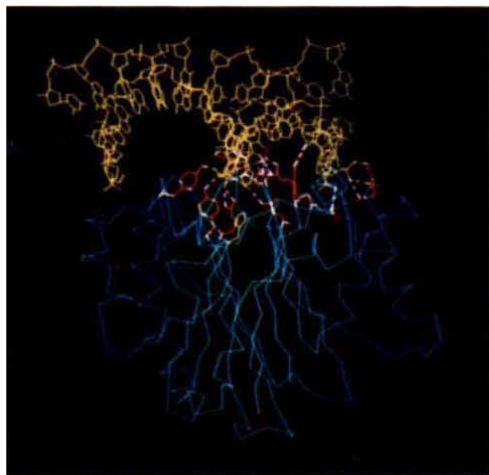
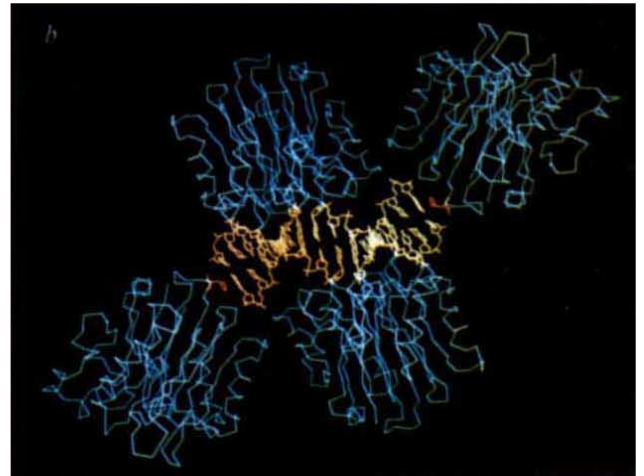


Fig. 2

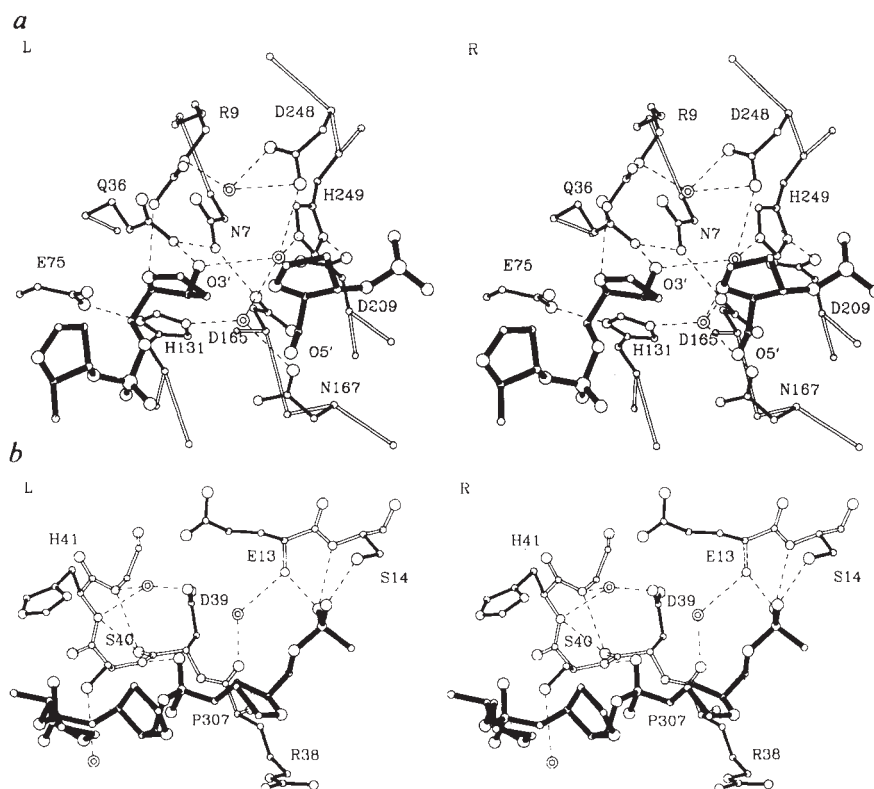
**Fig. 1** Formation of 14-mer and packing in the co-crystals. *a*, The octamer duplex is nicked in solution (arrow) and the dinucleoside diphosphate pGpC is removed from the 3'-end of one strand. On crystallization, the nicked double-strands dimerize across a 2-fold axis with base-pair formation between the single-stranded CG-regions to form a 14-mer with phosphates missing at two positions. *b*, Two nicked octamer duplexes (shown in orange and yellow) form a quasi-continuous 14-mer double-helix, roughly parallel to the *c*-axis, across a 2-fold rotation axis parallel *a*. The DNase I molecules (represented in blue by their C $\alpha$ -carbon backbone) at the upper left and the lower right interact in the minor grooves of the B-type DNA. The H118 residues (shown in red) of two other symmetry-related DNase I molecules stack on to 5'-terminal Gs of the 14-mer duplex.

**Fig. 2** Protein-DNA contacts in the DNase I-octanucleotide co-crystals. *a*, Stereoscopic views of the contacts between the 14-mer duplex and one DNase I molecule in increasing detail from top to bottom. These contacts occur in the minor groove and with phosphates from both strands, extending over 6 bp and two adjacent phosphates on one side of the double helix. Interacting in the minor groove are R38 (with the oxygen atoms of T305 and the neighbouring C306), Y73 of an exposed loop (with the deoxyribose ring of T313 in a stacking-type interaction) and R9 (with oxygen of C314). Six phosphates are in direct contact with the protein: two from one strand with backbone NH groups of E13, S14 and S40, four from the other strand with N71, R108 (salt-bridging), Y208 and S203. In intact DNA substrate a second salt-bridge would presumably be formed with R9, such that 7 phosphates would be in contact with DNase I. DNA is shown in yellow, except for the deoxyribose ring of T313 (purple), and DNase I residues interacting with DNA are depicted in red. Hydrogen bonds are indicated by broken lines, and the essential H131 in the immediate neighbourhood of the nick is drawn in green. *b*, Schematic drawing of the contacts between one DNase I molecule and the DNA-duplex. The one-letter code is used to represent protein residues; individual nucleotides (base, deoxyribose and 5'-phosphate group) are referred to in the text by the number in the circles representing the sugar rings. The position of the nick with no phosphate present and the crystallographic dyad are indicated. Nucleotides 301' and 302' belong to the 2-fold related molecule. The second cut which is induced by soaking Mn<sup>2+</sup> into the crystals (see text) occurs between C306 and G307.

groove for bulky groups. The stacking interaction of the 5'-terminal G with H118 of a symmetry-related DNase I molecule results from the packing in the C22<sub>1</sub> crystals and will not be present in solution.

What does the structure of this complex tell us about the mechanism of sequence-dependent DNA recognition by DNase I? The X-ray results confirm the assumption that the minor groove width is critical for the binding, and therefore most important in determining the cutting frequencies<sup>2,4</sup>. The narrow minor groove in regions with runs of As or Ts therefore nicely explains their low cleavage rates<sup>13,14</sup>. The groove width argument has also been used to explain the slightly lower-than-average cutting rates in G, C-rich regions, which are expected to have a wider-than-normal groove<sup>2,15</sup>. But in view of the 15Å-wide groove in the complex, these regions would be expected to be even more sensitive than random sequence DNA, and therefore this explanation is questionable. The observed bend in the DNA towards the major groove suggests that not only is the susceptibility of a given DNA stretch determined by the actual size of the groove, but also by its bendability or stiffness. An oligo(dA)/oligo(dT) track therefore shows low DNase I-sensitivity because of its narrow minor groove and a conformation rigidity that resists bending<sup>14</sup>. Furthermore, the bending induced by DNase I is in the opposite sense to that predicted by the Trifonov-Dieckmann wedge model for these sequences<sup>16,17</sup>. The stiffness of DNA has also been implicated in the specificity of interactions in repressor-operator complexes<sup>18</sup>. The effect of bending on DNase I sensitivity can be seen in looped or circularly closed DNA: compared to linear DNA of identical sequence, the susceptibility is enhanced in regions where the minor groove is facing outwards and is therefore widened, but is decreased where it faces the inside and is therefore compressed<sup>19,20</sup>. The observed bending in the DNase I-DNA complex provides a possible explanation for the so-called hypersensitive sites<sup>21</sup>: sequences bent either intrinsically or through the action of a bound protein towards the major groove would be particularly susceptible to DNase I degradation. But, the overall, slowly varying helix parameters, such as groove width and stiffness, do not explain the variation in cutting rates of

**Fig. 3** Protein environment of the two DNA cutting sites. *a*, Environment of the nick in the 14-mer-duplex. The essential H131 is located in the immediate neighbourhood of the expected position of the missing phosphate. The proposed acid-base catalysis of the nucleophilic attack by a water molecule through the E75-H131 pair<sup>4</sup> is possible, but H249 could have a catalytic function as well. Double circles represent water molecules, heavy lines indicate the DNA backbone. *b*, Environment of P307, the phosphodiester bond cleaved on soaking the co-crystals in Mn<sup>2+</sup>. S40 is in direct contact with this phosphate, side chains H41, E13, D39 and R38 are close by. This second active site is more than 15Å away from the essential H131.





neighbouring phosphodiester bonds<sup>1,2</sup>. Other local parameters must therefore also be involved which we are trying to define using a series of DNase I co-crystals with different DNA sequences (G. Frost and D.S., unpublished results).

The H131 residue is essential for the activity of DNase I (ref. 22) and is located at the cut in the complex. We have proposed a mechanism of acid-base catalysis for the nucleophilic attack of a water molecule by the E75-H131 pair<sup>4</sup> (Fig. 3) but definite conclusions cannot be drawn because the scissile bond is cut in solution and is not present in the crystal.

Divalent cations are required for DNA hydrolysis by DNase I (refs 23,24) and we were therefore surprised to find a cut oligonucleotide bound to the enzyme, despite a 1500-fold excess of EDTA over  $\text{Ca}^{2+}$  in the crystallization medium, although the reaction rate was at least  $10^4$  slower than with cations present. For staphylococcal nuclease the hydrolysis rate was accelerated by  $\text{Ca}^{2+}$  by a calculated factor of at least  $10^{4.6}$  (refs. 25). Also surprising was the detection of a second cut in the DNA, induced by diffusion of  $\text{Mn}^{2+}$  into the co-crystals. High pressure liquid chromatography of the dissolved crystals showed that the second cut did occur in the opposite strand, 4 bp away from the first nick (at P307, see Figs 2 and 3). This second site is more than 15 Å away from the first nick and the essential H131, and residues close to the cleaved phosphodiester bond include S40, E13, H41 and D39 (Fig. 3). The contacts to both sites involve the same DNase I molecule and are therefore independent of the crystal packing, so particular environment in the crystal is not responsible for the second cleavage. This indicates that DNase I contains a second,  $\text{Mn}^{2+}$ -activated catalytic site which we are now investigating using  $\text{Mn}^{2+}$ -impregnated DNase I-DNA co-crystals. A second active site could explain reports that DNase I induces double-strand breaks in DNA when activated by  $\text{Mn}^{2+}$  (refs 26-28).

We gratefully acknowledge the excellent technical assistance of Graham Frost and the advice of Heinz Bosshard, Johan

Postma and Paul Tucker on graphics display and film processing. We thank J. Priestley for an FFT-version of the PROLSQ refinement program.

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## Erratum

### Spatial heterogeneity: evolved behaviour or mathematical artefact?

John A. Downing

*Nature* **323**, 255-257 (1986)

LINE 4 in the legend to Fig. 1 in this letter should begin "(refs 17-37 and refs listed in ref. 4 as 3, 5, 6, ...)" In the original as printed the reader is referred to ref. 29 rather than ref. 4. □

## Corrigendum

### Synthesis and structure of (cis)-[1-ferrocenyl-2-(4-nitrophenyl)ethylene], an organotransition metal compound with a large second-order optical nonlinearity

Malcolm L. H. Green, Seth R. Marder, Mark E. Thompson, Judith A. Bandy, David Bloor, P. V. Kolinsky & R. J. Jones  
*Nature* **330**, 360-362 (1987)

THIS organotransition metal compound was thought to belong to the monoclinic space group Cc. Now the structure has been

re-refined in the orthorhombic space group F2dd, and this is found to be the correct space group using fewer parameters to define the model. In the original paper the crystal data section (Fig. 4 legend) should be replaced as follows:

$\text{C}_{18}\text{H}_{15}\text{FeNO}_2$ ,  $M = 333.2$ , orthorhombic, space group F2dd,  $a = 6.015(3)$ ,  $b = 44.547(10)$ ,  $c = 22.305(6)$  Å,  $U = 5,976.6$  Å<sup>3</sup>,  $z = 16$ ,  $D_c = 1.48$  mg m<sup>-3</sup>,  $\lambda(\text{Mo-K}\alpha_1) = 0.70930$  Å,  $\mu(\text{Mo K}\alpha_1) = 10.12$  cm<sup>-1</sup>,  $F(000) = 2,752$ . Refinement using full matrix least squares in the new spacegroup resulted in better agreement between observations and model. At convergence  $R = 0.037$ ,  $R_w = 0.040$  for 959 reflections with  $I > 3\sigma(I)$ . Iron, carbons of the cyclopentadienyl ligand (shown to exhibit marked anisotropic vibration), nitrogen and oxygen were refined anisotropically, remaining carbons isotropically and hydrogen in calculated positions, riding on the attached carbon. The cyclopentadienyl rings were refined subject to geometrical restraints.

In the original paper, the views of the molecular packing along the  $a$  axis and  $b$  axis were given. The molecular packing remains unaffected by the change of space group and the view along  $b$  differs from the original figure only by the redefinition of the  $c$  axis.

The authors thank Dr B. G. DeBoer for drawing attention to this matter. □

# Quantitative infectivity assay for HIV-1 and -2

P.L. Nara and P.J. Fischinger

*The development of a cytomorphologic infectivity microbioassay for human immunodeficiency virus (HIV) allows rapid, sensitive, and specific characterization of neutralizing antibodies or antiviral agents.*

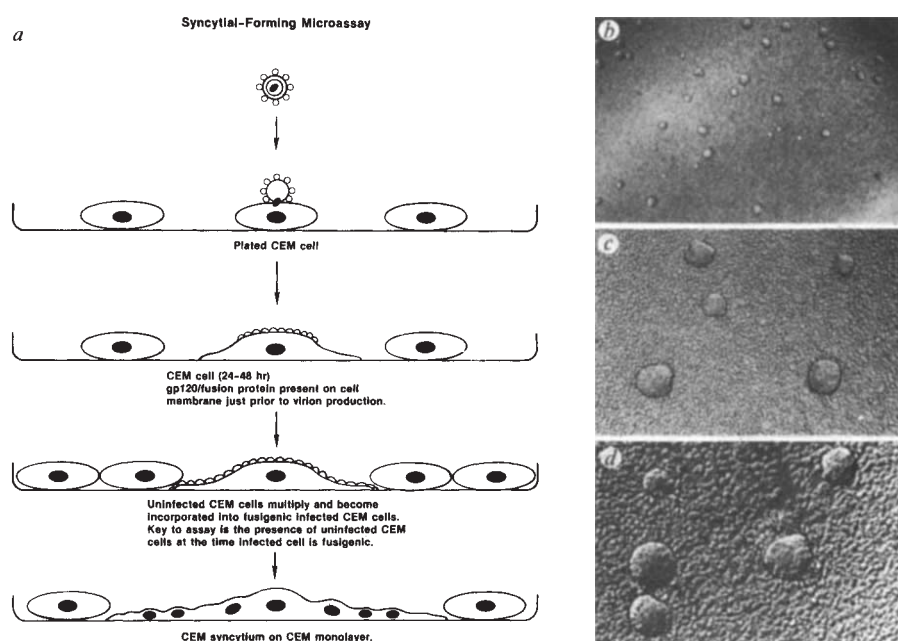
INFECTION with the human immunodeficiency virus (HIV) leads to a plethora of humoral and cellular immune responses<sup>1</sup>, which ultimately leads to AIDS in humans. The characterization and understanding of these immune responses in humans may allow for the identification of various states of protection from disease, opening up new avenues for future vaccine development and for interventional strategies such as antiviral agents and biological response modifiers. To this end, a dissection of host responses by sensitive, reproducible infectivity bioassays will be of obvious importance.

In the study of bacterial viruses, the production of plaques by single virus particles (bacteriophages) on a uniform bacterial layer was the earliest and most sensitive method for evaluating various anti-bacteriophage antisera<sup>2</sup>. Dulbecco introduced to animal virology a modification of the bacteriophage plaque assay that is now used and applied widely both for the quantitation of human and animal viruses and for neutralizing antibody<sup>3,4</sup>.

The virus neutralization/infectivity test is the most sensitive and specific serological procedure for demonstrating the quantitative relationship between virus and indicator cells. In such a test, a single infectious unit of virus infects a single cell and initiates a focal cytomorphologic expression of that infection, such as syncytium formation, plaque formation, cell transformation or lysis (Fig. 1). Because a single infectious unit leads to a single discrete response, a linear relationship exists between the number of virally-induced cytopathic effects and the first order of virus concentration<sup>3,4</sup>. When this test is applied in assays for virus inactivation or neutralization, the complex interactions of virus and antibody can be systematically and accurately evaluated<sup>4</sup>. The present assay<sup>5</sup> has been developed to provide a relatively rapid, quantitative, and reproducible bioassay for the detection and characterization of neutralizing antibody and other candidate antiviral agents.

## Syncytium-forming assay

The assay is based on the previously reported interaction between fusogenic virus-infected cells expressing the HIV envelope gene products and uninfected adjacent cells bearing CD4 molecules<sup>6</sup>. The conditions are optimized *in vitro* so that a virus inoculum introduced onto a syncytium-sensitive Leu 3a-positive CEM



**Fig. 1** *a*, Schematic of the CEM-SS virus-induced syncytium forming assay (reprinted with permission<sup>5</sup>). *b*, Microtitre well containing 35 typical HIV virally-induced syncytia. Approximately 30 per cent of the well is shown. *c*, Five HIV-induced syncytia embedded in a monomorphic CEM-SS monolayer. Magnification, 100 ×. *d*, Infectious cell centre assay. The five syncytia shown result from contact with virus-producing cells for 18 to 24 hours. Magnification, 200 ×.

cell line (CEM-SS) monolayer leads to a population of virus-infected (fusogenic) HIV envelope-expressing cells (Fig. 1*a*). These cells contact the remaining pre-titrated population of uninfected CEM-SS cells, allowing the early recruitment of uninfected cells into a detectable and quantifiable syncytium. With this method, agarose overlays are not needed because satellite syncytia from first- and second-generation virus progeny are not produced within the assay period.

In the assay, individual wells of a 96-well, flat-bottomed microtitre plate (Costar) are coated with 50 µl of a 50 µg ml<sup>-1</sup> poly-L-lysine solution (PLL) (Sigma) and allowed to stand at room temperature for 30 to 60 minutes, after which residual PLL is removed by two washes with physiologic buffered saline solution (PBS) or medium. During the PLL treatment of the microtitre plates, the non-adherent CEM-SS cells<sup>5</sup> are prepared for plating. To standardize plating conditions and assure a population of logarithmically growing cells, the CEM-SS cell cultures are split in a 1:2 ratio 24 hours prior to plating in RPMI 1640 medium containing 10 per cent fetal calf serum, one per cent antibiotics (PSN 100X Gibco Labs) and

0.5 per cent Nystatin (optional). The cells are counted, then gently pelleted at 800 r.p.m. for five minutes. The original volume of tissue culture medium is decanted and replaced with one-tenth the volume of complete RPMI 1640 medium containing 25 µg ml<sup>-1</sup> diethylaminoethyl-dextran (DEAE-D) for 30 minutes. DEAE-D may be omitted depending upon experimental conditions, but its presence assures maximum and more uniform viral kinetic infection of the cells. The cells are washed twice and resuspended in medium, and plated in the PLL-pretreated microtitre plates at a final cell concentration of 50,000 cells per well in a volume of 50 µl. Ten ml of 1.0 × 10<sup>6</sup> cells per ml is appropriate for one 96-well microtitre plate. The cells are allowed to attach for 30 minutes at 100 per cent humidity, 37 °C and five per cent CO<sub>2</sub>.

Frozen HTLV-III<sub>B</sub> and HTLV-III<sub>RF</sub> (isolates of HIV-1) stocks are made from recently infected H9 cells by centrifugation. The virus-containing media is aliquoted into 1 ml volumes and frozen at -70 °C. Viral stocks are then thawed and evaluated for syncytium forming units (SFU) by twofold endpoint dilution analysis in the CEM-SS assay. For confirma-



tion, tests for reverse transcriptase and p24 content by radioimmunoassay can be performed simultaneously. Other HIV variants — such as HTLV-MN, HTLV-CC and HIV-2<sub>NIH-Z</sub> — are labile and often lose titre on freezing, and must be maintained as freshly infected cultures. Optimal tissue culture conditions have been standardized, and reproducible virus yields can be achieved on a weekly basis and be titred directly in the assays.

Frozen, pre-titred or fresh virus stocks are diluted in medium to yield approximately 100 to 200 SFU per well. Fifty  $\mu$ l of approximately 100 SFU of virus (the constant component) is added to 50  $\mu$ l each of a serially prediluted sample of antiserum, purified immunoglobulin or other potentially virus-inactivating compounds under study. These virus-antibody mixtures are incubated for various times depending upon experimental conditions. Following incubation, each virus-antibody mixture is divided into duplicate wells (50  $\mu$ l per well) containing PLL-adherent CEM-SS cells (in which the medium has been removed gently) for 60 minutes. Following this adsorption phase, which lasts 60 minutes for most strains, the virus-media only and virus-antibody mixtures are removed and replaced with 100 to 200  $\mu$ l of media. Some wells of plated CEM-SS cells are left uninfected to serve as cytomorphologic controls. The microtitre plates are stored in an incubator at 100 per cent humidity, 37 °C and five per cent CO<sub>2</sub> for five days. On the third day, 100–200  $\mu$ l of complete medium may be added per well. On the fifth day, the plates are removed, the covers are removed, and a clear adhesive film (Plate Sealer, Linbro, Flow Labs) is placed over the microtitre wells. The covers then are replaced and each entire well can be counted for syncytia with an inverted microscope at a magnification of 40 to 100  $\times$  (Fig. 1b and c).

Initially, the uninfected CEM-SS cells are inspected for normal cell growth and viability, as determined by confluency of the monolayer at five days. This can serve as a toxicity marker when antiviral compounds are being investigated. Next, syncytia in all duplicate wells that received virus-media only are counted. This indicates if an adequate input of syncytium-forming virus was added to the assay system. The average number of syncytia obtained for the virus-media containing wells is designated  $V_o$ , for original virus inoculum. The syncytia then are counted in the duplicate wells that received the various virus-antibody mixtures. The average number obtained here for each serum dilution tested is designated  $V_n$ , for virus inoculum surviving after neutralization. After all of the wells have been counted for syncytia and have been averaged, the ratio of the number of syncytia induced by the virus inoculum surviving

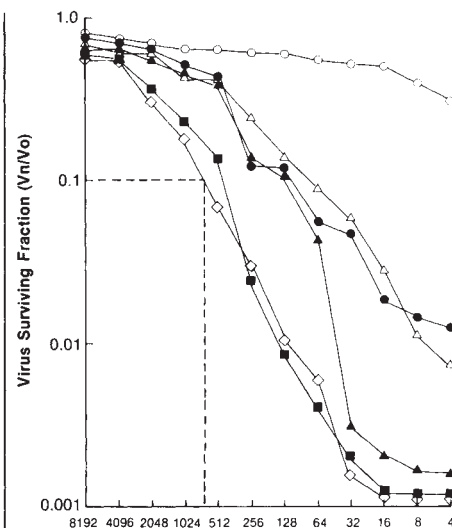
after virus neutralization ( $V_n$ ) to the number of syncytia induced by the virus inoculum ( $V_o$ ) is obtained. The data are represented as classical multiplicity curves<sup>1</sup>. The virus-surviving fractions ( $V_n/V_o$ ) from each twofold serum dilution are then plotted on the logarithmic Y axis, and the reciprocal of the serum dilutions are plotted on the arithmetic X axis. Each curve represents the virus-surviving fraction at progressive twofold serum dilutions (Fig. 2). Neutralization titres can be standardized at any level of per cent neutralization depending upon the individual experimental parameters. For HIV, a 90 per cent ( $V_n/V_o = 0.1$ ) point of virus inhibition is recommended as a standard neutralization index because of the variable initial slope of the curve at high serum dilutions.

Two minor modifications to the HIV-1 assay are necessary for HIV-2 because of the more rapid viral-infectivity replicative kinetics exhibited by HIV-2<sub>NIH-Z</sub> in this cell line. For HIV-2, 75,000 CEM-SS cells instead of 50,000 cells should be plated per well, and HIV-2-induced syncytia should be quantified on the third or fourth day instead of on the fifth or sixth day as previously described.

Several variants of simian immunodeficiency virus (SIV) have been tested with this assay to date. Several pathogenic strains of SIV/Delta have been found to replicate rapidly to maximal titres and to produce quantifiable syncytia in the CEM-SS cell line (M. Murphy-Corb, personal communication). The exact conditions of the assay are currently being defined, but the fact that some strains appear to work in the assay is encouraging. Two other strains, SIV/Mac and SIV/Mne, have been found to replicate to a lesser extent, and do not induce quantifiable syncytia formation (P. Johnson and R. Benveniste, personal communications, respectively).

### Infectious cell centre assay

The assay described above may be modified for the evaluation of infectious cell centres (ICC). Suspensions of cells that have been latently or productively infected by HIV-1 and -2 and produce virus or fusogenic proteins can be detected by this assay. The syncytia develop in 18 to 24 hours and are counted as described previously for HIV-1 (Fig. 1d). Specifically, CEM-SS cells are prepared and plated as described for HIV-1 and -2 infectivity assays, but a concentration of 100,000 cells per well is required. Cells to be quantified for virus production are washed three times in complete RPMI 1640 to remove any residual cell-free virus. The cells are then added to the previously plated adherent CEM-SS cells in tenfold incremental amounts ranging from 100 to 100,000 cells per well. In the evaluation of peripheral blood mononuclear cells from



**Fig. 2** Sequential analysis of neutralizing antibody response in an experimentally inoculated, persistently infected chimpanzee. The dashed line represents the 90 per cent neutralization ( $V_n/V_o = 0.1$ ) serum titre (512–1024) from a serum sample taken 2 yr ( $\diamond$ ) after inoculation (AI). Prebleed ( $\circ$ ), 1 month AI ( $\bullet$ ), 2 months AI ( $\triangle$ ), 3 months AI ( $\blacktriangle$ ), 1 yr AI ( $\blacksquare$ ).

infected humans, chimpanzees, or other primates, medium containing either interleukin-2 or phytohaemagglutinin — used to support growth and viral expression — do not adversely affect the CEM-SS cells.

Isolates of HIV-1 and -2 other than those reported here need to be evaluated by twofold, endpoint-limiting dilution-amplification analysis<sup>5</sup> for their quantitative relationship to syncytia formation and the detection of non-syncytial variants. The isolates used here have been found to be free of non-syncytial variants. Should non-syncytial variants exist with other isolates, they can be evaluated in this assay system by removing supernatants and cells for viral antigen detection by radioimmunoassay, reverse transcriptase or immunofluorescence assays.

The CEM-SS virus-induced, syncytium-forming microassay should provide AIDS researchers with a rapid, quantitative, reproducible bioassay for the sensitive evaluation of multiple samples for antiviral or neutralizing activity against HIV-1, -2, and some SIV strains.  $\square$

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# Latest products for AIDS research

*The study of acquired immune deficiency syndrome (AIDS) crosses the boundaries between immunology, virology and molecular biology. Several products for AIDS research are described below.*

INTERNATIONAL Enzymes Inc. offers a line of **antibodies, antigen preparations and antisera** for AIDS research (*Reader Service No. 101*). Affinity purified sheep antibodies to HIV envelope and structural core proteins are available, including antibodies to gp120, gp41, p24-I, II, III (combined), p24-I, p24-II, p24-III and p18. Each antibody comes in 0.5 mg, 1 mg and 2 mg quantities, costing \$200, \$350 and \$680 (US), respectively. The antibodies are lyophilized in PBS without preservatives, and may also be purchased conjugated with fluorescein isothiocyanate or alkaline phosphatase. International Enzymes sells HTLV-I and HIV antigen for \$1,250 (US) per mg, and human plasma positive for HIV antibody for \$850 (US) per litre. Synthetic gp120 and gp41 peptide-coated 96-well plates are also available for \$180 (US) per plate.

For cleaning up laboratory spills safely, Day-Impex is distributing a **virucidal disinfectant** named Virkon (*Reader Service No. 102*). Day-Impex says Virkon wipes out human and animal viruses from all



The Virkon sachets from Day-Impex are a handy size for small spills.

of the seventeen known viral families, including HIV, hepatitis A and B, herpes and polio. Virkon also eliminates bacteria, fungi, yeasts and moulds. Virkon is composed of a blend of peroxygen compounds, surfactant, organic acids and an inorganic buffer system, and has a pH of 2.6 in solution. It is non-toxic to humans, virtually odourless and leaves no harmful residues in the environment, says Day-Impex. Virkon is sold for £60 (UK) for 50 sachets of 50 g each, and is used as a one per cent solution.

A free **colour wallchart** from Becton Dickinson graphically illustrates the relationships between human T-cell and natural killer cell subpopulations (*Reader Service No. 103*). The chart represents the

primary reactivity of monoclonal antibodies with lymphocyte subpopulations in normal human peripheral blood, as determined by two- and three-colour immunofluorescence staining and flow cytometric analysis.

## Monoclonals

Cellular Products Inc. sells murine **monoclonal antibodies to HIV** for the detection of core and envelope proteins of the HIV virus in viral infected cell cultures using immunoprecipitation or Western blot techniques (*Reader Service No. 104*). Monoclonal antibodies to p17 (subclass IgG<sub>2a</sub>), gp160/gp41 (subclass IgG<sub>1</sub>) and p24 (subclass IgG<sub>1</sub>) are available. The antibodies are purified using protein A, agarose and ion-exchange chromatography, and Cellular Products says they are stable for nearly three months at 2-8 °C. The antibodies are sold in 100 µg vials for \$175 (US).

A monoclonal antibody to the HIV *gag* gene protein product, p24, is available from Cytotech (*Reader Service No. 105*). The **anti-p24 antibody** may be used in Western blot applications, immunofluorescence, ELISA techniques, or to purify HIV p24 protein by immunoaffinity chromatography. The monoclonal antibody is sold in a 100 µl size for \$110 (US) and in a 250 µl size for \$250 (US).

T Cell Sciences offers murine monoclonal antibodies for various human T-cell activation antigens. The company's ACT-T-SET TLiSA1 recognizes TLiSA1, a T lineage specific activation antigen expressed on the surface of activated T-cells which reaches maximal expression at 3-6 days after *in vitro* stimulation, and which



The T Cell Sciences range of antibodies to T-cell activation antigens includes the monoclonal TLiSA1.

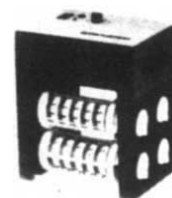
remains highly expressed for several weeks (*Reader Service No. 106*). T Cell Sciences says studies have indicated that the addition of an antibody which binds to TLiSA1 in mixed lymphocyte cultures markedly inhibits the progression of cytolytic T lymphocyte (CTL) precursors to

functional CTL while having no effect on cell proliferation. The ACT-T-SET TLiSA1 monoclonal antibody is offered in a liquid form as either purified antibody or FITC conjugate, and is packaged in 100 test configurations. T Cell Sciences includes a collection of research reports and abstracts with the product.

Bioproducts for Science, Inc. is distributing a series of monoclonal antibodies for the study of **human leukocyte cell surface markers**, through an agreement with Serotec Ltd (*Reader Service No. 107*). The Campath series are highly characterized rat monoclonal antibodies. Because ascites can be produced more economically in

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rats than in mice, Campath antibodies are competitively priced. Bioproducts for Science says the Campath antibodies can be substituted in every application where mouse monoclonals are currently used, and that rat monoclonals may recognize epitopes and antigens on cell surfaces different from those recognized by antibodies derived from mouse hybridomas. Additionally, antibodies from rat IgG<sub>2b</sub> can elicit antibody-dependent cell-mediated toxicity induced by human K-cells, and certain rat monoclonals are able to lyse cells with homologous complement. Each vial of 1 mg of total protein in 0.5 ml of diluent contains enough antibody for 200 tests when using a second step reagent for immunofluorescence.

## Kits

A division of SmithKline Beckman, SmithKline Bio-Science Laboratories, recently introduced its Hivagen **HIV antibody test** as a confirmatory test to replace the Western blot assay in the diagnosis of HIV infection (*Reader Service No. 108*). SmithKline Bio-Science Laboratories says the ELISA-format test had no false positives in clinical trials, and that it will dramatically reduce the amount of inconclusive HIV antibody test results. The Hivagen test uses a panel of six pure, recombinant viral proteins, eliminating the false positive results caused by other human proteins that have been exhibited by tests derived from whole virus preparations. The recombinant proteins include: Kp120CC, Kp120N and Kp41, derived from genes for the envelope proteins gp120 and gp140; Kp24 and Kp55, from core proteins p24 and p55; and Kp66/31, a polymerase peptide derived from a combination of polymerase proteins which include protease, endonuclease and

transcriptase. The cost of Hivagen — \$80 (US) per test — is claimed to be competitive with that of Western blot confirmation tests.

Coulter Immunology offers an **assay for HIV antigen** that it says can detect 1.0–1.56 pg of antigen in a 200 µl well sample, in less than four hours (*Reader Service No. 109*). The assay uses a murine monoclonal antibody to capture HIV p24 core protein, which is recognized by a human polyclonal biotin antibody probe and detected through the reaction of biotin with a streptavidin-conjugated horseradish peroxidase system. The assay works with either serum, plasma or culture fluid. Coulter says the assay has no cross-reactivity with HTLV-I, HTLV-II, CMV, herpes simplex I and II, Epstein-Barr virus, Legionnaires', toxoplasmosis, rheumatoid factor or bilirubin. A lysing reagent destroys viral infectivity in the sample, rendering any virus present totally non-infectious after 15 seconds. Coulter sells the HIV antigen assay in a 96-well kit, containing 12 strips of eight wells each, for \$528 (US). Neutralization reagent is available separately for the confirmation of positive samples.

AgriTech Systems will soon be introducing diagnostic and research tests for **feline T-cell lymphotropic lentivirus (FTLV)** (*Reader Service No. 110*). The feline T-cell lymphotropic lentivirus causes immunosuppression in cats, and feline retroviral infection may be evaluated as a model system for HIV infection in humans. The test will be based on the FTLV discovered in 1986 by Niels C. Pedersen and Janet Yamamoto of the University of California at Davis. AgriTech Systems hopes to have the tests on the market within several months.

## Equipment

The Microlab **liquid dispenser** from Hamilton, distributed by V.A. Howe & Company Ltd, increases the safety of pipetting HIV samples (*Reader Service No. 111*). Hamilton's dispenser is meant for assays that require samples to be transferred from tube to tube, or from tube to plate. The unit uses a single syringe that is controlled by a microprocessor so that a sample can be aspirated from a tube and



The MicroLab M dispenser from Hamilton and V.A. Howe.

then be dispensed — with or without reagent — into another tube or microwell plate. The Microlab M handles volumes from 1 µl up to 25 ml, and multiple or single reagents can be added with a controlled dilution rate. Every method used can be stored in memory, increasing the reproducibility of results. Two steps may be performed at once, cutting down on assay drift. The Microlab M costs £2,690 (UK).

Dynatech's **Easywell plate viewer** helps take the guesswork out of microplate pipetting (*Reader Service No. 112*). The Easywell helps distinguish between the wells of 96-well microplates or microwell strips that have already been filled with liquid, and those that are still empty. The Easywell is based on an illuminated grid network which is observed through the bottom of the wells. The sample itself acts as a marker: when a sample solution has been added, the network is greatly enlarged in round-bottom wells and decreased in flat-bottom wells. Dynatech says the Easywell aids in the dispensing of standard and control samples in the same microplate, because it makes it easier to keep track of the border in which samples are added. □

*These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.*

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